CELLULAR MECHANISMS OF VESTIBULAR COMPENSATION- AN *IN VITRO* STUDY

by

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For my grandfather

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DECLARATION

The work carried out in this thesis was carried out by myself, apart from the experiments in part 2 of chapter 3 which were completed in conjunction with Dr. Toshiaki Yamanaka. This work was conducted under the supervision of Dr M. B. Dutia in the Department of Physiology, at the University of Edinburgh between September 1995 and August 1998.

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CONTENTS **page**

Introduction

The maintenance of a stable direction of gaze is essential in many mammalian behaviours, such as visuo-motor co-ordination, spatially-orientated tasks and visual tracking of objects in the external world. In recent years the reflex systems controlling head and eye movements have been studied extensively, both at the peripheral and central levels (Wilson and Melvill-Jones, 1979; Carpenter, 1988; Peterson and Richmond, 1988; Berthoz *et al.*, 1992; Peterson *et al.*, 1992; Wilson, 1993; Dieterich and Brandt, 1995; Dulac *et al.*, 1995; Wilson *et al.*, 1995). Movements of the head in space are detected by the vestibular, optokinetic and cervical proprioceptive afferent systems, their patterns of activity encode the direction and velocity of head displacement. These afferent inputs give rise to a family of inter-related reflexes, whose primary functions are to stabilise the eyes within the head and the head in relation to the trunk (Dutia, 1989).

Two major reflexes arise from vestibular afferent inputs, the vestibular-ocular reflexes (VORs), which help to stabilise the retinal image during head rotation by rotating the eyes in the orbits by a nearly equal and opposite amount to the head rotation (Wilson and Melvill-Jones, 1979; Dutia, 1989; Berthoz *et al.*, 1992; Dieterich and Brandt, 1995); and vestibulo-collic reflexes (VCRs), which give rise to the reflex excitation of the appropriate group of neck muscles to counteract the head displacement, thus acting to stabilise the head on the trunk (Wilson and Melvill-Jones, 1979; Dutia, 1989; Berthoz *et al.*, 1992; Wilson *et al.*, 1995). These reflexes are elicited by inputs from the semicircular canals and otolith afferents of the inner ear, which project, via the VIIIth cranial nerve, to the vestibular nuclei in the brainstem (figs. 1.1 and 1.2). The medial vestibular nucleus receives afferents mainly from the horizontal semicircular canal, while afferents from the anterior and posterior canals project to the superior vestibular nucleus (Wilson and Melvill-Jones, 1979). Thus second-order cells in the medial vestibular nucleus are involved mainly in mediating the horizontal VOR, while those in the superior vestibular nucleus are responsible for the VORs in the vertical plane. Vestibular nucleus neurones project in turn either

rostrally to the abducens nuclei and the oculomotor nuclei, or caudally to the neck muscle motoneurone pools in the cervical spinal cord (Wilson and Melvill-Jones, 1979; Carpenter, 1988; Buttner-Ennerver, 1992).

Of the four principle vestibular nuclei (the inferior, lateral, medial and superior nuclei, Fig 1.2), the lateral and medial vestibular nuclei (MVN) are the most extensively studied. The MVN is the largest and most easily identified vestibular nucleus in the freshly dissected brain, and has proved to be an attractive target for the application of brain-slice techniques to study the electrophysiology and neuropharmacology of vestibular neurones (for reviews see, Smith and Darlington, 1995; de Waele *et al.*, 1995; Vibert *et al.*, 1997).

The vestibular-related synergies stabilizing gaze and posture in the horizontal plane display important plastic properties and the process of "vestibular compensation" is an excellent model for the study of lesion-induced plasticity in the adult central nervous system. Vestibular compensation is a process of behavioural recovery which occurs in animals following damage to one vestibular labyrinth or nerve (unilateral labyrinthectomy, UL), after which a severe syndrome of ocular motor and postural symptoms develop. These symptoms are usually divided into *static* and *dynamic* symptoms, depending upon whether they persist in the absence of head movement (static) or occur as a result of head movement (dynamic). *In vivo* studies using electrophysiological techniques have demonstrated that the static symptoms observed during the acute stage after UL correspond to an imbalance in the resting discharge rate of vestibular neurones between the intact and lesioned sides of the brain. Remarkably, the static symptoms of UL recover rapidly in most mammalian species within only a few days after UL. This behavioural recovery is associated with a rebalancing of the resting discharge rate of neurones in the intact and lesioned vestibular nuclei (Smith and Darlington, 1991; Darlington *et al.*, 1992; Curthoys and Halmagyi, 1995; Dieringer, 1995; Darlington *et al.*, 1995; Vibert *et al.*, 1997; Lacour, 1998).

An enormous volume of research has been published on the subject of vestibular compensation since the original work of Flourens and Bechtrew in the late 1800's,

including electrophysiological, biochemical and morphological changes which take place after UL. A number of ideas have been postulated as to the mechanisms involved in the process of vestibular compensation, including upregulation of excitatory postsynaptic receptors, substitution of non-vestibular sensory inputs and reactive synaptogenesis (reviewed in: Smith and Darlington, 1991; Darlington *et al.*, 1992; Curthoys and Halmagyi, 1995; Dieringer, 1995; Darlington and Smith, 1995; Vibert *et al.*, 1997; Lacour, 1998). However, to date, the cellular and molecular mechanisms which underlie vestibular compensation are still unknown.

The work described in this thesis represents one of the first systematic studies of the cellular mechanisms involved in the early stages of vestibular compensation in the MVN in the adult rat.

VESTIBULAR FUNCTION

1.1 THE MEDIAL VESTIBULAR NUCLEUS AND ITS CONNECTIONS

1.1.1 Afferent innervation of the Medial Vestibular Nucleus

Impulses originating from the hair cells of the vestibular labyrinth are transmitted along the vestibular nerve afferent fibres which terminate in the vestibular nuclei in the brainstem. The vestibular nerve contains afferents from the three semicircular canals, the utricle and the saccule. Anatomical studies using axon degeneration or anterograde labelling techniques have demonstrated that the vestibular nucleus complex (VNC) receives an endorgan-specific distribution of primary vestibular nerve afferents, leading to the suggestion that each vestibular nucleus is involved in a separate reflex pathway. Degeneration studies have shown that vestibular afferent fibres terminate in all of the nuclei, although not in all areas (Walberg *et al.*, 1958; Brodal, 1974). The rostral area of the medial vestibular nucleus (MVN) receives a particularly dense innervation of primary afferents from the ipsilateral semicircular canals and in particular, the horizontal semicircular canal (Stein and Carpenter, 1967; Gacek, 1969; Korte, 1979; Carleton and Carpenter, 1984; Sato *et al.*, 1989). Precht and Shimazu (1965) were the first to demonstrate that cells in the MVN of the cat were activated monosynaptically and polysynaptically by electrical stimulation of the vestibular nerve. Wilson *et al.* (1967) subsequently showed that cells responding monosynaptically to vestibular nerve stimulation were located mainly in the rostral portion of the MVN. In conjunction with this, many neurones in the rostral area of the MVN respond to angular acceleration of the head in both the horizontal and vertical planes (Shimazu and Precht, 1965; Markham, 1968; Curthoys and Markham, 1971).

In the cat MVN, four types of neuronal responses have been identified in response to horizontal rotational vestibular stimulation (Duensing and Shaffer, 1958; Shimazu and Precht, 1965).

Type I neurones increase their discharge frequency with acceleration to the ipsilateral side and show a decrease with contralateral acceleration.

Type II neurones decrease their discharge frequency with ipsilateral acceleration and show an increase with contralateral acceleration.

Type III neurones increase their discharge frequency with acceleration in either direction.

Type IV neurones decrease their discharge frequency with acceleration in either direction.

Of 300 vestibular neurones recorded *in vivo* in the decerbrate cat by Shimazu and Precht (1965), 67% of neurones studied showed a type I response, 29% a type II response, 3% a type III response, and only 1% a type IV response. Melvill Jones and Milsum (1970), showed similar proportions of type I and type II responses recorded *in vivo* in the cat. However, in the awake monkey Fuchs and Kimm (1975), showed equal proportions of type I and II responses.

Shimazu and Precht (1965) subdivided type I neuronal responses into two categories, *tonic* and *kinetic*. According to their classification, kinetic neurones comprised 20% of the type I cells and were characterised by:

i) no resting discharge.

ii) monosynaptic excitation by ipsilateral vestibular nerve stimulation.

iii) an increase or decrease in their discharge frequencies along steep slopes during horizontal rotational vestibular stimulation.

In contrast, tonic neurones made up 80% of the cell population recorded and were characterised by,

i) spontaneous activity at rest.

ii) polysynaptic activation in response to a single electrical shock to the ipsilateral vestibular nerve.

iii) less steep response sensitivities to activation or cessation of horizontal rotational vestibular stimulation.

However, in studies where sinusoidal stimuli have been employed (which may reveal the tonic or kinetic activation of the neural responses better than the transient stimuli used in the previous studies), there is no evidence for the existence of tonic or kinetic units, and therefore the classification of second-order type I MVN neurones into tonic and kinetic subtypes is debatable (Carpenter, 1988).

1.1.2 Commissural connections

Primary vestibular afferents do not project to the vestibular nuclei on the contralateral side (Brodal, 1974). The first report of a vestibular commissural pathway was by Ramon y Cajal (1909), who described it as consisting of crossed vestibular root fibres. Ladpli and Brodal (1968), were the first to demonstrate the topology and importance of the vestibular commissure as one of the major contingents of vestibular efferents. Using axonal degeneration techniques and retrograde axoplasmic transport techniques it has been demonstrated that a commissural pathway exists interconnecting the cells of all the bilateral vestibular nuclei with the exception of the LVN (Fig 1.2, Ladpli and Brodal, 1968; Gacek, 1978; Pompeiano *et al.*, 1978; Carleton and Carpenter, 1983; Ito *et al.*, 1985; Epema *et al.*, 1988). The neural basis of this commissural pathway was first studied by Shimazu and Precht (1966), who showed that when the contralateral nerve is electrically stimulated, field potentials can be recorded in the ipsilateral vestibular nuclei. This response was abolished following a midline incision which interrupted the commissural fibres, confirming that the contralateral labyrinth influences the activity of the ipsilateral vestibular nuclei through the commissural fibres (Shimazu and Precht, 1966). In mammals, stimulation of the contralateral vestibular nerve is predominantly inhibitory on the ipsilateral side (Shimazu and Precht 1966: Precht *et al* 1973b; Kasahara and Uchino 1974), whereas in amphibians it is mainly excitatory (Ozawa *et al.*, 1974; Knopfel 1987; Cochran *et al.*, 1987; Dieringer, 1995).

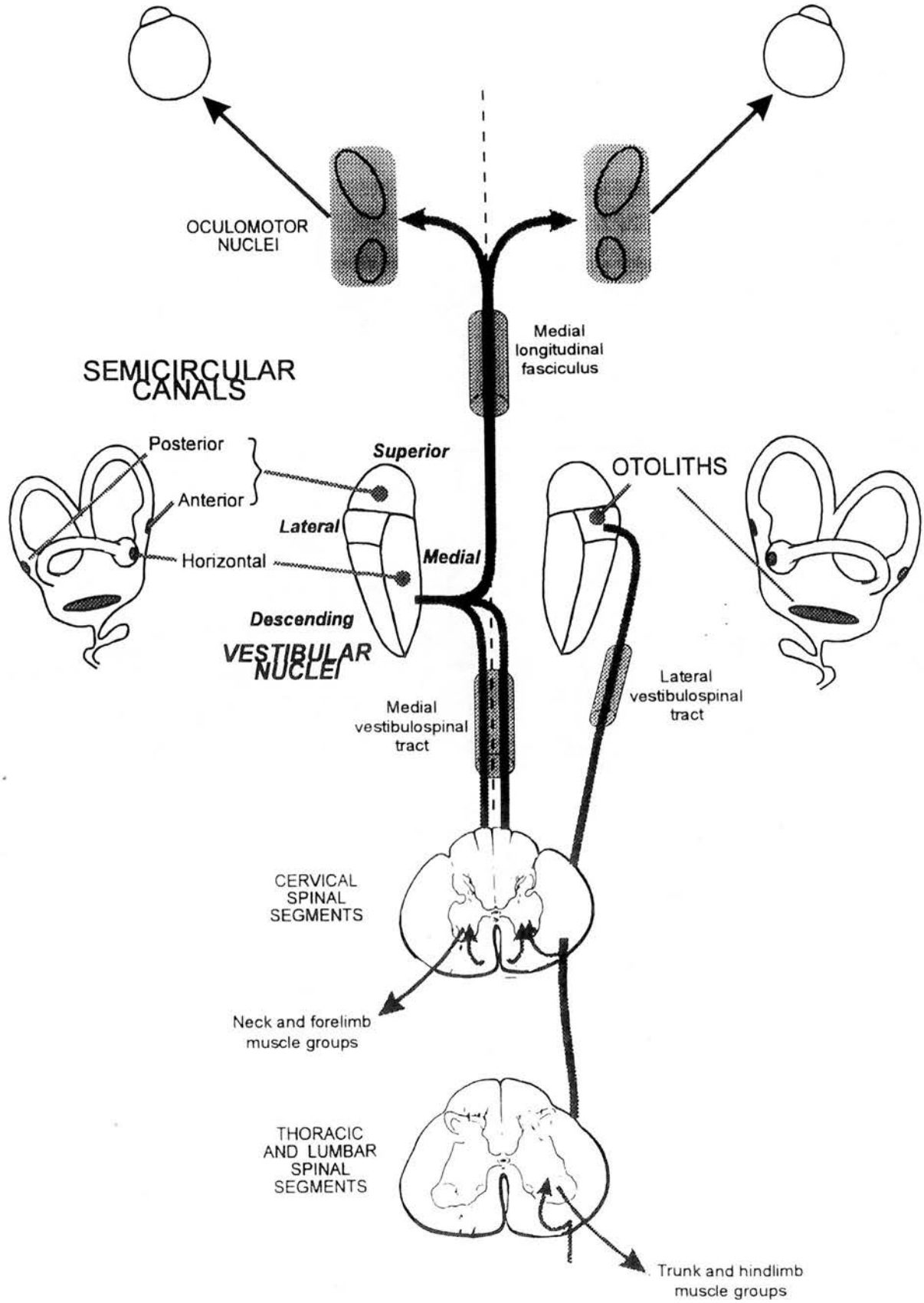


Figure 1.1

Schematic diagram of the labyrinthine afferent inputs to the vestibular nuclei, and the rostral and caudal projections arising from the medial vestibular nucleus.

1.1.3 Efferent projections from the MVN

Vestibulo-ocular pathways

The output from vestibular nuclei neurones is relayed by short latency pathways to the neck, trunk, limbs and extrocular motoneurones. Anatomical studies, using degeneration methods and retrograde transport of horseradish peroxidase (HRP) injected into individual extraocular muscles, have shown that the main vestibulo-ocular projection originates in the MVN and superior vestibular nucleus (SVN), and ascends in the medial longitudinal fasciculus (MLF) to the motoneurone pool of the oculomotor nuclei (Fig 1.1, McMasters *et al.*, 1966; Tarlov, 1970; Gacek, 1971, 1974, 1977; Isu and Yokota, 1983).

The extraocular muscles of each eye are innervated by three groups of motoneurones whose cell bodies are located in specific brainstem nuclei (Tarlov and Tarlov, 1971; Gacek, 1974; Graf and Ezure, 1986). The motoneurones of the abducens nucleus innervate the ipsilateral rectus muscle whereas the contralateral superior oblique muscle is innervated by motoneurones in the trochlear nucleus. The remaining four muscles, the superior rectus, inferior rectus, medial rectus and inferior oblique are innervated by motoneurones from the oculomotor nucleus. Axons from the MVN enter the abducens and oculomotor nuclei bilaterally, whereas they project only ipsilaterally to the trochlear nucleus (Fig 1.2. Wilson and Melvill Jones, 1979).

Spino-vestibular pathways

A primary afferent projection from the neck to the MVN and descending vestibular nucleus (DVN) in mammals has been demonstrated both histologically (Corbin, 1937; Edney and Porter, 1986; Prihoda *et al.*, 1991), and electrophysiologically, and MVN neurones have been found to be activated after stimulation of the cervical afferents (Mergner *et al.*, 1982). In mammals, three vestibulospinal projections have been established: a lateral vestibulospinal tract, a medial vestibulospinal tract and a caudal vestibulospinal tract (Fig 1.2. Wilson and Jones, 1979; Sijolung and Bjorklund 1983; Hata and Watanabe, 1990).

The lateral vestibulospinal tract originates in the lateral vestibular nucleus and projects to the ipsilateral extensor muscle motoneurone pools (Brodal, 1962, 1964; Carpenter, 1960). The medial vestibulospinal tract originates primarily in the medial vestibular nucleus, but physiological studies have shown that the lateral and descending nuclei also contribute to this pathway (Akaike, 1973; Akaike *et al.*, 1973) sending their fibres within the MLF to the cervical enlargement (McMasters *et al.*, 1966; Nyberg-Hansen, 1964). The caudal vestibulospinal tract originates in the lateral vestibular nucleus and parts of the medial and descending vestibular nucleus (Peterson *et al.*, 1978).

1.1.4 Intrinsic connections

Using axonal degeneration techniques Ladpli and Brodal (1968), demonstrated the presence of intrinsic connections between the ipsilateral vestibular nuclei which were first suggested by Cajal (1909) and Lorente de No (1933). More recent studies using retrograde and anterograde axoplasmic transport techniques have confirmed the existence of these intrinsic connections (Rubertone *et al.*, 1983; Carleton and Carpenter 1983; Carpenter and Cowie 1985; Ito *et al.*, 1985; Epema *et al.*, 1988). Epema *et al* (1988), demonstrated that the MVN projects reciprocally to the other three vestibular nuclei. Apart from connections between the individual subnuclei, there are reciprocal connections between the rostral and caudal MVN (Pompeiano *et al.*, 1978; Epema *et al.*, 1988).

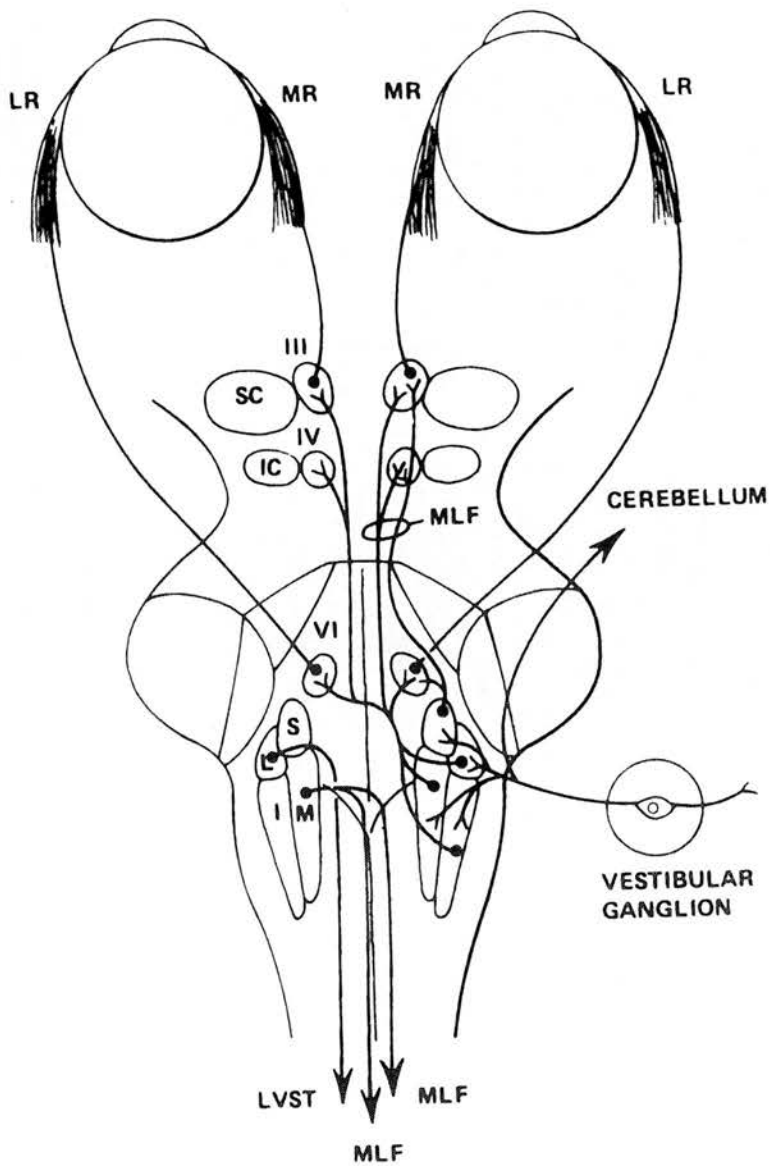


Figure 1.2

Schematic illustration of the main projections arising from the vestibular nuclei. The origins of the descending projections are illustrated on the left side of the diagram and the origins of the ascending projections on the right side. Only the connections to the medial (MR) and lateral (LR) rectus muscles which control horizontal gaze are shown. III, oculomotor nucleus; SC, superior colliculus; IV, trochlear nucleus; IC, inferior colliculus; MLF, medial longitudinal fasciculus; VI, abducens nucleus; S, superior vestibular nucleus; L, lateral vestibular nucleus; I, inferior vestibular nucleus; M, medial vestibular nucleus; LVST, lateral vestibulo-spinal tract.
This illustration has been adapted from Gilman and Newman (1992).

1.1.5 The Cerebellum

The activity of the vestibular nuclei is continually under the influence of the cerebellum. The area involved is known as the *vestibulocerebellum,* and consists of the flocculus, nodulus, uvula, and ventral paraflocculus. The flocculus receives vestibular (Brodal and Hoivik, 1964), visual (Maekawa and Simpson, 1973), neck (Wilson *et al.*,1967), and extraocular proprioceptive afferents (Kimura and Maekawa, 1981), projects to the vestibular nuclei (Anguat and Brodal, 1967: Yamamoto and Shimoyama, 1977; Balaban, 1984), the lateral cerebellar nucleus and to the perihypoglossal nuclei (Yamamoto, 1979). The nodulus receives vestibular (Brodal, 1974; Precht et al., 1976a), visual (Maekawa and Simpson, 1973), and neck proprioceptive afferents (Precht et al., 1976b), and projects to the vestibular and fastigial nuclei. Axons from the nodulus terminate in the caudal potion of the MVN, whereas fibres from the flocculus end in the rostral MVN (Brodal, 1974: Anguat and Brodal, 1967).

Recent anatomical studies (Gerrits and Voogd, 1987; Nagao, 1992, Nagao *et al.,* 1997; Glickstein *et al.*, 1994), have shown that the rostral half of the monkey flocculus exhibits different anatomical features from the caudal portion. The caudal half receives mossy fibre projections mainly from the vestibular system, whereas the rostral half receives mossy fibre projections predominantly from the pontine nuclei. These observations are consistent with the suggestion by Gerrits and Voogd (1987), that the caudal half of the monkey flocculus is equivalent to the flocculus of other species, e.g. cat, rabbit, and the rostral half is equivalent to the ventral paraflocculus. The monkey ventral paraflocculus receives mossy fibre inputs mainly from the nucleus reticularis tegmenti pontis and pontine nuclei (Gerrits and Voogd., 1987; Nagao, 1992; Glickstein *et al.*, 1994), but little input from the vestibular system (Nagao, 1992). Outputs from the ventral paraflocculus terminate in the cerebellar interpositus, dentate nucleus and the vestibular complex (Nagao *et al.*, 1997).

Vestibular primary afferent projection to the cerebellum

In both the rat and cat, Dow (1936), showed that the uvula-nodulus, but not the flocculus or paraflocculus receive a vestibular primary afferent projection. A direct projection from the vestibular nerve to the flocculus in monkeys (Carleton and Carpenter, 1984; Nagao *et al.*, 1997) and cats (Brodal and Hoivik, 1964), was demonstrated using retrograde axonal transport and autoradiography techniques. These results were disputed by Gerrits *et al.* (1987), who reported that the vestibular projection to the ipsilateral flocculus was virtually absent in rabbits, an observation which was also noted by Barmack *et al.* (1993). This study, using double-labelling techniques, also revealed that there was a vast vestibular primary afferent projection to the uvula and nodulus, almost as great as the afferent projection to the vestibular nuclei. The mossy fibre projection was found to be exclusively unilateral. It is possible then that species differences occur with respect to the vestibular projection to the cerebellum.

Cerebellar nuclei-vestibular projections

The deep cerebellar nuclei, which include the dentate and fastigial nuclei, send projections to the MVN. The more caudal portion of the fastigial nucleus projects to the contralateral MVN whereas the rostral region projects to the ipsilateral nuclei (Ito, 1970). These fibres exert an excitatory effect on MVN neurones (Ito *et al.*, 1970; Schimazu and Smith, 1971), whereas the Purkinjie cells of the flocculus and nodulus have an inhibitory action (Fukuda *et al.*, 1972; Baker *et al.*, 1972).

Vestibulo -cerebellar nuclei projection

Studies using HRP have shown that primary vestibular neurones and second order axons project to the fastigial nuclei, however there is no evidence for primary vestibular neurones terminating in the dentate nucleus (Kotchabhakdi and Walberg, 1978a). Electrophysiological evidence agrees with the above anatomical studies in that fastigial neurones can be influenced by stimulation of the vestibular nerve or labyrinth (Furuya *et al.*, 1975), as can neurones in the nodulus and flocculus (Precht and Linas,

1969; Shinoda and Yoshida, 1975).

1.1.6 The Inferior Olive

Anatomical and physiological studies (see De Zeeuw 1994, for review), have shown that the vestibular nuclei affect the activity of neurones in the inferior olive (IO). This pathway carries information to specific olivary subnuclei. Histological (Ramon y Cajal, 1909) and ultrastructural investigations of the inferior olive have determined that the IO is composed of three major subdivisions the principal olive, the medial, and dorsal accessory olive and four minor subdivisions, the nucleus beta, the dorsal cap, the ventrolateral outgrowth and the dorsomedial cell column.

Inferior olive and vestibulocerebellum

The IO is the sole source of cerebellar climbing fibres which terminate upon Purkinje cells (Desclin, 1974). HRP injected into the flocculus labelled cells contralaterally in the dorsal cap and the ventrolateral outgrowth (Yamamoto, 1979; Takeda and Maekawa, 1980; Maekawa *et al.*, 1989), while HRP injected in the nodulus labelled neurones in the dorsal cap, ventrolateral outgrowth and substantial numbers in the beta nucleus (Bernard, 1987; Maekawa *et al.*, 1989). These studies suggest that collateralized climbing fibres might project to both the flocculus and the nodulus. In a more recent study, Kaufman *et al.*, (1996), used a modified form of the pseudorabies virus to trace the pathways arising from areas of the cerebellum to the inferior olive. Twenty-four hours after an injection into the left flocculus and ventral paraflocculus, virus-infected neurones were labelled contralaterally in the dorsal cap, ventrolateral outgrowth and medial accessory olive. At the same time following an injection into uvula and nodulus, virus-infected neurones were located in the contralateral beta nucleus. Some virus-infected neurones were also located in the MVN, DVN and prepositus hypoglossi at this time (Kaufman *et al.*, 1996). These tracing studies are consistent with the earlier studies of Yamamoto, (1979), Bernard, (1987), and Maekawa *et al.*, (1989).

Inferior olive and cerebellar nuclei

From anatomical studies it is evident that the cerebellar nuclei and the IO are strongly and specifically interconnected (Ruigrok, 1997). However, the nucleo-olivary pathway is GABAergic (Angaut and Sotelo, 1989; Fredette and Mugnaini, 1991), whereas the olivary climbing fibres are excitatory to the Purkinje cells and to their target cells in the cerebellar nuclei (Kitai *et al.*, 1977). The olivary projection to the cerebellar nuclei originates as climbing fibre collaterals and is excitatory. The cerebellar projection to the IO is exactly reciprocal to the olivonuclear projection, but in addition, also has a variable ipsilateral component, and has been demonstrated to be GABAergic (Ruigrok, 1997).

Inferior olive and vestibular nuclei

To date, there are few studies on direct connections between the MVN and IO, however recently it has been shown that MVN neurones project to the beta nucleus of the inferior olive (De Zeeuw, 1994).

1.2 IN VITRO STUDIES OF THE MEDIAL VESTIBULAR NUCLEUS

Using extracellular and intracellular recording techniques, Gallagher *et al.* (1985) were the first to demonstrate that rat MVN neurones fired spontaneous action potentials in a brain slice preparation of the rostral medulla containing the MVN. They further showed that in their *in vitro* preparation, MVN cells fired at discharge rates similar to those observed *in vivo* (Precht *et al.*, 1966; Hamann and Lannou, 1988; Smith and Curthoys, 1988a,b Newlands and Perachio, 1990a,b;). Since 1985, numerous *in vitro* studies, using both extracellular and intracellular recording techniques have confirmed the findings of Gallagher and colleagues that MVN neurones are spontaneously active when maintained *in vitro*, firing at discharge rates ranging from 0.5-60 spikes/s, with a mean firing frequency between 10-25 spikes/s (Lewis *et al.*, 1987; Ujihara *et al.*, 1989; Doi *et al.*, 1990; Dutia *et al.*, 1992; Johnston *et al.*, 1992, 1993, 1996; Darlington *et al.*, 1989, 1993, 1995; Smith *et al.*, 1990; Smith and Darlington, 1992; Serafin *et al.*,1991, 1992, 1993). These observations were initially surprising since it was generally assumed that a large component of the resting activity of MVN neurones *in vivo* is provided by excitatory input from the vestibular nerve.

In vitro studies have consistently demonstrated that a large component of the spontaneous activity is generated by an intrinsic mechanism, as it persists after the blockade of synaptic transmission with calcium channel blocking agents or by perfusing the slice with high Mg^{2+} ,low Ca^{2+} aCSF (Gallagher *et al.*, 1985, 1992; Darlington *et al.*, 1989; Dutia *et al.*, 1992). Intracellular studies have shown that the MVN spontaneous discharge rate is highly voltage-dependent (Serafin *et al.*, 1991a,b; Gallagher *et al.*, 1992; Johnston *et al.*, 1994) and is sensitive to both changes in pH and temperature (Fukuda and Loeschcke, 1977; Kobayashi and Murakami, 1982).

From their intracellular studies in the rat, Gallagher *et al.* (1985) identified two groups of spontaneously active MVN neurones *in vitro*, which were distinguished by their afterhyperpolarisation (AHP) profiles. One cell group showed a single AHP following the spike, the other a double AHP (Fig 1.3). In a series of intracellular investigations in guinea pig brainstem slices containing the MVN, Serafin and

colleagues identified three main types of MVN neurones on the basis of their AHP profiles (Serafin *et al.*, 1991a,b). Type 'A' MVN neurones were characterised by having a single deep AHP and constituted 30% of the population of spontaneously active neurones recorded in their study. This group also displayed a 4-aminopyridine-resistant A-like rectification and small, high-threshold calcium spikes. In contrast, Type 'B' MVN neurones displayed a double AHP, an initial fast and small component, followed by a delayed and slower component. Type 'B' cells also displayed large, high-threshold calcium spikes and comprised 50% of MVN cells recorded. A third, non-homogenous class of cells were named Type 'C'. These neurones were an intermediate cell type and could not be classed as type A or B and so were not studied in detail. Vibert *et al.* (1997) recently stated that, "type C cells may represent a true subpopulation of MVN neurones, or a pool of cells whose properties were distorted by intracellular impalement"

The classification of rat MVN neurones by Gallagher *et al.* (1985) was recently confirmed in a more detailed intracellular study by Johnston *et al.* (1994). Here it was reported that Type 'A' neurones (33% of the neurones sampled) had a single, deep AHP which was mediated by a tetra-ethyl ammonium (TEA)-sensitive K^+ conductance and an apamin-insensitive Ca^{2+}-activated K^+ conductance. Type 'B' neurones (67% of the sample) had an early, fast AHP which was mediated by a TEA-sensitive K^+ conductance and a delayed, slow AHP mediated by an apamin-sensitive Ca^{2+}-activated K^+ conductance (Fig 1.3).

To date, little is known about the functional connections of these cell types. Experiments using intracellular dye filling techniques and tracing the axons of type A and type B cells may indicate whether the axons travel rostrally in the medial longitudinal fasciculus towards the oculomotor nuclei or caudally in the direction of the spinal cord or in both directions, indicating whether cell types correspond to vestibulo-ocular, or vestibulo-collic cells. Although many *in vitro* MVN studies have identified the receptor subtypes mediating the excitatory or inhibitory effects of most

of the known endogenous neurotransmitters present in the CNS (for review see de Waele *et al.*, 1995), no differences have been found between type 'A' and type 'B' cell responses to transmitters have been reported so far.

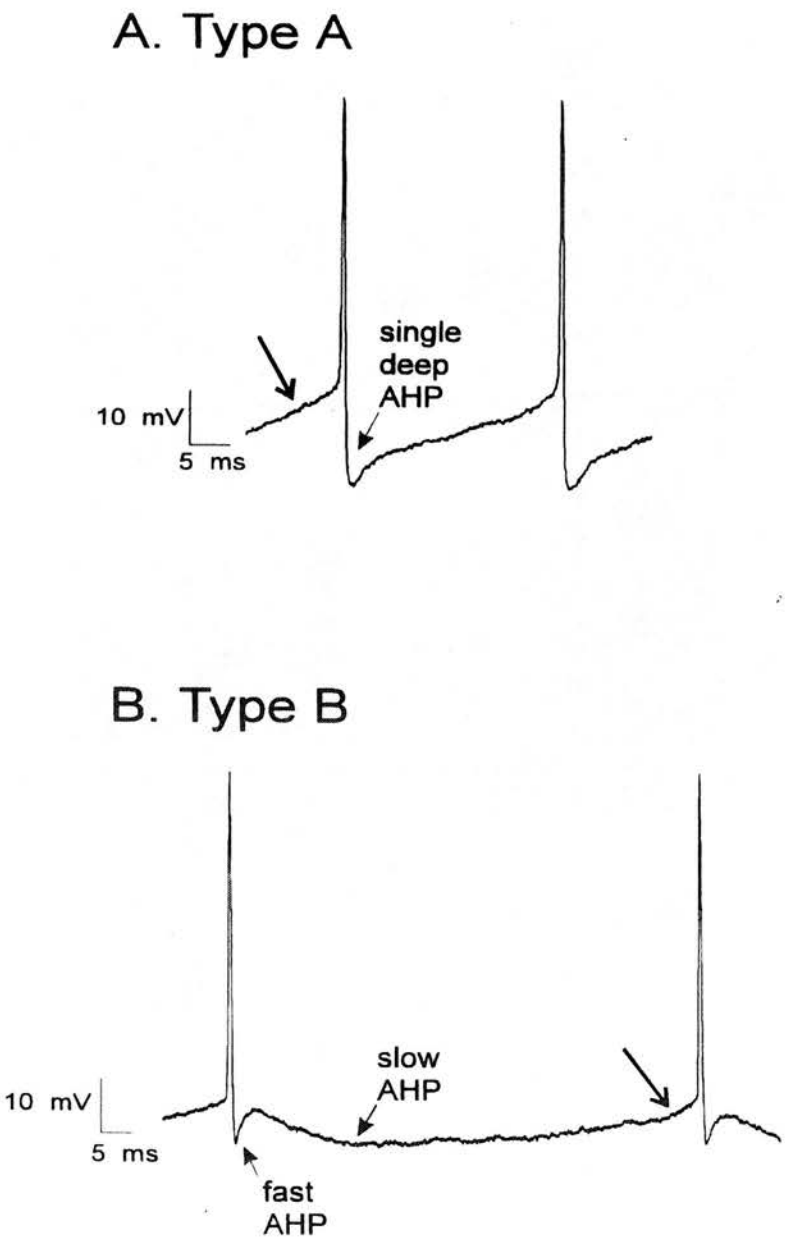


Figure 1.3

Spontaneous action potentials of cell types A and B present in the rat MVN arise from a gradual membrane depolarisation preceding the spike (arrows). Results obtained using intracellular recording techniques.

A. a Type 'A' cell showing the single deep AHP

B. a Type 'B' cell showing both fast and slow AHPs

This illustration has been adapted from the PHD thesis of A.R. Johnston (1995) University Of Edinburgh

1.3 NEUROTRANSMITTERS AND NEUROMODULATORS OF THE VESTIBULAR SYSTEM

Behavioural, electrophysiological and biochemical methods have been used to study the pharmacology of the central vestibular pathways. Electrophysiological studies have been performed both *in vivo*, and with the development of the brain slice preparation containing the MVN, *in vitro*. Morphological studies using immunocytochemical and autoradiographic methods have also contributed significantly to the understanding of the neurochemistry of the vestibular system (fig 1.4) (see Zanni *et al.*, 1995; de Waele *et al.*, 1995; Smith and Darlington, 1996, for recent reviews).

1.3.1 Neurotransmitters actions on vestibular neurones

Excitatory amino acids

Early studies suggested that acetylcholine was the neurotransmitter involved in synaptic transmission between primary vestibular afferents and second-order vestibular neurones (Matsuoka *et al.*, 1985). However, this hypothesis has now been discarded and it is now believed likely that an excitatory amino acid (glutamate or aspartate) may be involved. Raymond *et al.*(1984) reported that the presynaptic terminals of vestibular nerve fibres took up glutamate, and that this glutamate uptake was significantly decreased after vestibular nerve section. Since then, a large number of immunocytochemical and autoradiographic studies have supported this idea. Several studies have provided evidence that the vestibular nerve is immunoreactive for glutamate (Reichenberger and Dieringer, 1994; Straka *et al.*, 1995), and recent *in situ* hybridisation, immunohistochemical and reverse transcription-polymerase chain reaction (RT-PCR) studies have provided evidence for the presence of mRNA for the NMDA receptor subunit NR1 in the vestibular ganglion (Niedzielski, 1995; Fujita, 1994). Ligand-binding studies have shown that there is a high level of glutamate receptors in the vestibular nuclei, especially in the MVN (Toutai *et al.*, 1989, Raymond *et al.*, 1989). In addition, recent electrophysiological studies have shown that glutamate is released when the vestibular nerve is electrically stimulated (Yamanaka *et*

al., 1995, 1997). Synaptic responses evoked in second order MVN neurones by electrical stimulation of the root of the eighth nerve were found to be blocked by application of glutamate (non-NMDA) antagonists (Cochran, 1987; Lewis *et al.*, 1987, 1989; Doi *et al.*, 1990; Gallagher *et al.*, 1992; Carpenter and Hori, 1992; Capocchi *et al.*, 1992; Kinney *et al.*, 1993, 1994; Peusener and Giaume, 1994; Takahashi *et al.*, 1994a; Straka and Dieringer 1993, 1996; Straka *et al.*, 1996). Excitatory postsynaptic potentials (EPSPs) evoked by stimulation of the eighth nerve remained unchanged in Mg^{2+} free aCSF (Lewis *et al.*, 1987, 1989; Cochran *et al.*, 1987), suggesting that although MVN neurones posses NMDA receptors, the synapse between the vestibular nerve and 2nd order neurones is mediated by non-NMDA subtype of glutamate receptor. Together these experiments indicate that synaptic transmission between primary vestibular afferents and second order neurones is exclusively glutamatergic and is via non-NMDA receptor subtypes.

However, recent whole-cell patch-clamp studies, using slices from young rats (postnatal days 4-6), reported that EPSP's induced in MVN neurones by electrical stimulation of the eighth nerve can be blocked by NMDA receptor antagonists at high levels of depolarisation (Takahashi *et al.*, 1994a). These findings were supported by Kinney *et al.*, (1994), using similar aged rats (postnatal days 7-25). Furthermore, Ca^{2+}-fluoresence studies (also using young rats) have shown that the increase in $[Ca^{2+}]_i$ that occurs in MVN neurones following vestibular nerve stimulation can be reduced by NMDA receptor antagonists (Takahashi *et al.*,1994b).

The discrepancy between the two groups of data may reflect the difference in the age of rats used, as there are developmental differences in the expression of the NMDA receptor. Developmental differences of NMDA receptor function have been demonstrated in the visual cortex (Kato and Yoshimura, 1990), and hippocampus (Tremblay *et al.*, 1988), where NMDA receptors have been shown to posses a reduced Mg^{2+} block in young (<25 days postnatal) rats. It is possible then that NMDA receptors in the MVN are more active and involved in synaptic transmission during an early period of postnatal development becoming relatively inactive during maturation,

this would then account for the observations of Takahashi *et al*, (1994a), and Kinney *et al*. (1994).

The functions of NMDA receptors within the VNC remain uncertain, but it has been suggested that they mediate inputs from the spinal cord and commissural input from the contralateral VNC in frogs. For example, in the isolated frog medulla it has been demonstrated (Knopfel 1987, Cochran *et al*., 1987), that the EPSP recorded intracellularly, from ipsilateral MVN neurones evoked by electrical stimulation of the commissural fibres from the contralateral MVN, contained slow-rising EPSP's which could be blocked by NMDA receptor antagonists. This suggests that the commissural pathway in the frog is mediated by glutamate acting on NMDA receptors. In the rat however, it appears that both NMDA and non-NMDA receptor subtypes mediate excitatory synaptic transmission from the contralateral MVN (Doi 1990).

Inhibitory amino acids

In mammals, the extensive commissural system, connecting the vestibular nuclei and the nucleus prepositus hypoglossi, uses both GABA and glycine as neurotransmitters (Precht *et al*., 1973a; Furuya *et al*., 1991). The tonic discharge rate of second order vestibular neurones in mammals is inhibited by electrical stimulation of the contralateral vestibular nerve through the commissural fibre system linking the vestibular nuclei. Precht *et al*. (1973a,b) showed that a systemic injection of strychnine, a selective antagonist of glycine receptors, reduced commisural inhibition *in vivo*. This inhibition was also blocked by $GABA_A$ antagonists indicating that both GABA and glycine receptors mediate commissural inhibition of type I MVN neurones. In a later study, iontophoretically applied glycine *in vivo* inhibited the spontaneous discharge of 82% of cat second order vestibular neurones recorded extracellularly from the MVN (Furuya *et al*., 1991): however, commissural inhibition was abolished only by $GABA_A$ antagonists but only suppressed by strychnine. (Furuya *et al*., 1991, 1998). The differences between the two groups may be due to differences in experimental protocols. In the experiments by Precht *et al*. (1973b) antagonists were administered

systemically and therefore may have had actions on other areas of the CNS as well as the vestibular commissures.

1.3.2 Neuromodulators of central vestibular neurones

Excitatory amino acids

Pharmacological studies and studies using cloning technology have demonstrated that the CNS contains at least two groups of glutamatergic receptors: ionotropic receptors, including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxalone propionate (AMPA) and Kainate subtypes of the glutamate receptor, and the G-protein coupled metabotropic glutamate receptors (mGluRs) (for review see Nakanishi, 1992). A large body of evidence has demonstrated that the central vestibular nuclei possess all these different subtypes of glutamate receptors.

Using *in vitro* brainstem slices containing the MVN of the guinea pig, application of NMDA receptor antagonists decreased the resting discharge rate of MVN neurones when recorded extracellulary (Smith *et al.*, 1990; Smith and Darlington, 1992). Serafin *et al.* (1992) found that MVN neurones recorded intracellularly from guinea pig brainstem slices were depolarised by application of NMDA, and this effect could be blocked by the NMDA antagonist D,L-APV, indicating that glutamate depolarises MVN neurones via NMDA receptors. Application of NMDA increased the spontaneous discharge and was associated with a decrease in membrane resistance. Similar observations were recorded by Lewis *et al.*, (1987, 1989), Gallagher *et al.*, (1985), Smith and Darlington, (1992). However, in the study by Doi *et al.*, (1990), only a small number of MVN neurones were affected by the application of the NMDA antagonist, D,L-APV (Doi, 1990).

Although studies of the metabotropic glutamate receptor in the VNC are limited at present, it seems likely that this receptor subtype also makes a critical contribution to VNC neuronal function (Vibert *et al.*, 1992; Kinney *et al.*, 1993; Peterson *et al.*, 1995; Darlington and Smith, 1995). Morphological studies have shown that vestibular

neurones express metabotropic receptors of the mGluR1, mGluR2, mGluR5 and mGluR7 subtypes (Shigemoto *et al.*, 1992; Romano *et al.*, 1995; Ohishi *et al.*, 1995; Neki *et al.*, 1996).

There are few behavioural studies which reveal a role for glutamate receptors in vestibular function. De Waele *et al.* (1990) were the first to demonstrate that a unilateral injection of the NMDA receptor antagonist, D,L-APV, but not the kainate/AMPA receptor antagonist, CNQX, into the vestibular complex of rats resulted in ocular motor and postural deficits usually associated with a unilateral labyrinthectomy. More recent studies (Furuya *et al.*, 1996) *in vivo* have shown that NMDA receptor antagonists block excitation of type I MVN neurones by NMDA and other glutamate agonists and are consistent with the theory that NMDA receptors in the VNC may make a contribution to VOR function.

Inhibitory amino acids

The role of GABA as an inhibitory neurotransmitter within the VNC has been known since the early work of Curtis *et al.* (1970). All vestibular nuclei are densely innervated by GABAergic and glycinergic afferent fibres (Kumoi 1987; Nakao 1982), and immunocytochemical studies have revealed the presence of immunoreactivity for GABA or glutamic acid decarboxylase (GAD) an enzyme specific for GABA synthesis, in neurones in the VNC of several species (Blessing 1987; Dupont 1990; Kumoi 1987; Nomura 1984). Using immunocytochemical techniques, Holstein *et al.* (1992) demonstrated the presence of small and medium-sized GABA neurones throughout the VNC, being more numerous in the MVN and LVN. In the MVN, the density of staining appeared to be greatest rostrally. *In situ* hybridization studies have shown that vestibular neurones express high amounts of $GABA_A$ and glycine receptors while both presynaptic and postsynaptic $GABA_B$ receptors were described in the MVN by immunocytochemical techniques (Holstein *et al.*,1992). Glycine may also have a role in vestibular information processing in mammals. Immunohistochemical and immunocytochemical studies have demonstrated the presence of numerous glycinergic cells and fibres in the MVN (Pourcho *et al.*, 1992; Spencer *et al.*, 1989, 1992), and a

subpopulation of primary vestibular afferents fibres exhibit glycine-immunoreactivity (Reichenberger *et al.*, 1993). The strychnine-sensitive glycine receptor has been localized on MVN neurones by autoradiography (Zarbin *et al.*, 1981), immunocytochemistry (Araki *et al.*, 1988), and *in situ* hybridization techniques (Sato *et al.*, 1991).

In vitro application of GABA, or specific GABA agonists, inhibits the tonic activity of and hyperpolarises the membrane of MVN neurones and this action can be antagonised by the selective $GABA_A$ antagonist, bicuculline (Gallagher *et al.*, 1985; Smith *et al.*, 1991; Dutia *et al.*, 1992; Vibert *et al.*, 1995). Smith *et al.*, (1991), reported that in the guinea pig, only 50% of neurones tested were responsive to GABA and, in a more recent study, fewer than 50% of neurones tested with the selective $GABA_A$ receptor agonist, isoguvacine were inhibited. However, Dutia *et al.* (1992) demonstrated that all rat MVN neurones were inhibited by GABA. This inhibition was blocked by antagonists of $GABA_A$ and $GABA_B$ receptor subtypes, indicating that both subtypes of the GABA receptor are involved in regulating the spontaneous discharge of MVN neurones. The differences between the two groups of studies are probably due to the different slice thickness used in the two studies (Dutia *et al.*,1992). Dutia *et al.* (1992) found that low micromolar concentrations of GABA were ineffective on cells tested in slices thicker than 350μM, but inhibited cells in slices 200-250μM thick. The slices used in the study by Smith *et al.* (1991) ranged between 400-600μM. Recent studies by Vibert *et al.* (1995) have provided further evidence for the presence of $GABA_B$ receptors in the MVN. Using intracellular recording techniques they demonstrated that both type A and type B MVN neurones were hyperpolarised by application of GABA or the specific $GABA_B$ agonist, baclofen. *In vitro* application of glycine inhibited 50% of MVN neurones in guinea-pig brainstem slices (Smith *et al.*, 1991); in a more recent study, Lapeyre *et al.* (1995) showed that all neurones tested with glycine were inhibited in a dose-dependent manner; the inhibition persisted when synaptic transmission was blocked, suggesting that the site of action is postsynaptic.

The functional significance of GABA receptors within VOR pathways has recently been investigated. Unilateral injection of the $GABA_A$ agonist, muscimol, into the MVN caused a labyrinthine syndrome directed to the ipsilateral side and severe deficits in gaze-holding (Mettens, 1994). $GABA_B$ receptors have been implicated in ocular motor intergration, since the $GABA_B$ receptor agonist, baclofen, impairs gaze-holding and the VOR time constant (Nikalasson, 1994).

The question arises as to whether GABA and glycine modulate different functions within the VNC. Neurones in the MVN that project to the spinal cord are immunoreactive for GAD (Blessing *et al.,* 1987), but other evidence suggests that glycine is involved in these vestibulospinal connections. For example, Felpel (1972) demonstrated that intravenous injection of strychnine, but not GABA antagonists, depressed the amplitude of the disynaptic IPSP recorded in neck muscle motoneurones following a single electrical stimulus to the vestibular nerve *in vivo* in the cat. These experiments suggest that the transmitter involved in the caudally projecting VCR pathway is glycine.

Histamine

The central histamine systems have received considerable attention for their possible role in vestibular-related disturbances, such as vertigo and motion sickness, indeed anti-histamine drugs are commonly used in the treatment of such disorders. However, the precise action of histamine within the VNC is unclear with contradictions existing between the available *in vivo* and *in vitro* data.

A direct projection from the histamine neurones of the tuberomammilary nucleus in the posterior hypothalamus to the VNC have been described in rats (Takeda *et al.*, 1986; Panula *et al.*, 1989; Steinbusch and Mulder, 1986), guinea pigs (Airakinsen and Panula, 1988), rabbits (Iwase *et al.*, 1993), and cats (Tighilet and Lacour, 1996, 1997). Ligand binding studies have demonstrated a high density of the histamine H1 receptor in the MVN (Bouthenet *et al.*, 1988). Subsequent injections of retrograde tracers into this nucleus have shown bilateral neuronal staining localized in the posterior

hypothalamus (Takeda *et al.*, 1986; Lacour, 1988). Early *in vivo* electrophysiological studies by Kirsten and colleagues (1976b), reported that the majority of MVN and LVN neurones exhibited a decrease in tonic discharge rate in response to ionophoretically applied histamine. The H_1 antagonist, diphenhydramine, failed to block the response to histamine, whereas the response was blocked by the H_2 receptor antagonist metiamide. In contrast, *in vitro* electrophysiological studies using intracellular recording techniques have demonstrated that histamine predominantly depolarises the membrane potential and increases the tonic discharge rate of MVN neurones. These effects could be reproduced by the H_2 receptor agonist, impromidine (Phelan *et al.*, 1990; Serafin *et al.*, 1992) and inhibited by H_2 antagonists. Recently however, using extracellular recording techniques, histamine has been shown to excite rat MVN neurones by activating both H_1 and H_2 receptor linked mechanisms (Inverarity *et al.*, 1993; Wang and Dutia, 1995).

In the guinea pig, postural and/or oculomotor changes, similar to those observed after a unilateral labyrinthectomy, have been reported after infusion of impromidine (H_2 agonist), α-methylhistamine (H_3 agonist), cimetidine (H_2 antagonist) or thioperamide (H_3 antagonist) into the vestibular nuclei (De Waele *et al.*, 1993; Yabe *et al.*,1993; Lacour, 1988). Unilateral infusion of histamine agonists and antagonists thereby creates an imbalance in activity between the vestibular nuclei comparable to that induced by unilateral labyrinthectomy.

5-hydroxytryptamine

The VN receives serotonin or 5-hydroxytryptamine (5-HT)-containing projections from the dorsal raphe nucleus, and immunohistochemical studies have also demonstrated 5-HT-containing cells in the MVN (Steinbusch 1981, 1991). Recent ligand-binding studies have demonstrated 5-HT_{1A}, 5-HT_{1B} and 5-HT_2 receptors on VN neurones with the highest densities in the rostral portion of the MVN (Zanni, 1995). The response of MVN neurones to iontophoresis of 5-HT receptor agonists and antagonists has been examined *in vivo*, where both increases and decreases in discharge

rate were observed in rat MVN neurones (Licata *et al.*, 1993). However, *in vitro* electrophysiological studies have demonstrated most MVN neurones are excited by bath application of 5-HT. Application of 5-HT to rat MVN neurones recorded intracellulary *in vitro* depolarised the membrane potential (Gallagher *et al.*, 1992), an observation which was also made by Vibert *et al.* (1994). Using extracellular recording techniques from MVN neurones *in vitro*, it has been demonstrated that 5-HT excites most MVN neurones through a 5-HT_2 receptor-linked mechanism, and inhibits a minority of cells through a 5-HT_{1A} receptor-linked mechanism (Johnston *et al.*, 1992; 1993). Very few behavioural studies have investigated the role of serotonin on vestibular function. However, intracerebroventricular injection of 5-HT has been shown to increase the amplitude of the VOR in the alert rat (Ternaux and Gambarelli, 1987).

Dopamine

The application of dopamine *in vitro* caused depolarisation of MVN neurones in the rat (Gallagher *et al.*, 1992) and guinea-pig (Vibert *et al.*, 1995b). This depolarisation was mimicked by selective D_2 receptor agonists but not by D_1 receptor agonists. In the same study, it was shown that when synaptic transmission was blocked by a low Ca^{2+}, high Mg^{2+} media, dopamine had a weak postsynaptic, hyperpolarizing action on all MVN neurones, which was suggested to occur via a D_2-receptor.

Noradrenaline

Recent immunohistochemical studies have demonstrated that noradrenergic fibres project from the locus coeruleus to all subnuclei of the vestibular nuclei (Schuerger and Balaban 1993; Hozawa *et al.*, 1993). Using receptor autoradiographic techniques, Zanni *et al.* (1995) demonstrated the presence of $alpha_2$-adrenergic receptors in the VNC with the most intense staining seen in the caudal portion of the MVN, while the highest receptor density was seen at the dorsal border. *In vivo* electrophysiological studies have shown that iontophoretically injected noradrenaline produced excitation of LVN neurones but inhibited MVN neurones (Yamaoto 1967; Kirsten and Sharma

1976b). *In vitro* studies have reported that noradrenaline depolarizes MVN neurones (Gallagher *et al.*, 1992), whereas Vibert *et al.*, (1994), reported that bath application of noradrenaline caused both depolarization and hyperpolarization of MVN neurones. The explanation for the discrepancy between *in vivo* and *in vitro* data remains unclear, but may be due to different receptor subtypes being activated at different concentrations of applied agonist.

Acetylcholine

Immunocytochemical and autoradiographic studies have revealed the presence of the acetylcholine synthesing enzyme (cholineacetyltransferase), and the acetylcholine inactivating enzyme (acetylcholinesterase) in the VNC and vestibular nerve, and the detection of a high density of both muscarinic and nicotinic cholinergic receptors in the MVN (Clarke *et al.*, 1985, Swanson *et al.*, 1987). Ionotophoretic application of acetylcholine *in vivo* increases the firing rate of LVN and MVN neurones, and this effect is blocked by the muscarinic antagonist atropine, but not by nicotinic antagonists (Kirsten and Schoner, 1973; Kirsten and Sharmer, 1976a,b). *In vitro* electrophysiological studies agree with data from *in vivo* studies that a muscarinic cholinergic receptor mediated mechanism is involved in modulating the tonic discharge of MVN neurones (Ujihara, 1989; Dutia *et al.*, 1990; Carpenter and Hori, 1992). Dutia *et al.* (1990) added that the muscarinic acetylcholine receptor involved is not of the M_1-subtype. Intracellular recordings from MVN neurones have demonstrated that cholinergic agonists predominantly depolarise the membrane potential of MVN neurones through both muscarinic and nicotinic cholinergic receptor-linked mechanisms (Phelan and Gallagher, 1992). These effects persisted during the block of synaptic transmission, suggesting that they are produced postsynaptically.

Peptide receptors

Numerous neuropeptides have been identified throughout the CNS, and most of their actions are through specific metabotropic receptors. A number of neuropeptides may act as neuromodulators of the central vestibular neurones, including substance P,

the opioid peptides, somatostatin and adrenocorticotrophin (for review see Balaban 1989).

The opiate peptides

Numerous studies have demonstrated the presence of enkephalinergic neurones and terminals in the vestibular nuclei, and binding studies have demonstrated moderate levels of opiate binding sites, with the highest level being reported in the MVN (Beitz *et al.*, 1987; Nomura *et al.*, 1984; Zanni *et al.*, 1995). Electrophysiological studies have provided conflicting evidence as to the actions of opiates in the vestibular nuclei. Carpenter and Hori (1992, 1994), reported that the majority (30%) of tonically active rat MVN neurones tested iontophoretically *in vitro* were excited both by morphine and ala-leu-enkephalin. The excitation was inhibited by the opioid antagonist, naloxone, implicating both μ and δ opiate receptor subtypes in this response. The responses to opioid receptor agonists persisted during the blockade of synaptic transmission, indicating that the opioid receptors are located postsynaptically. In contrast, studies by Kawabata *et al.* (1990) and Sulaiman and Dutia, (1998) have shown inhibitory effects of opiate agonists in the MVN. Using iontophoretic application of enkephalin *in vivo* Kawabata *et al.* (1990) demonstrated that the increase in resting discharge rate normally exhibited by MVN type I neurones during horizontal rotation to the ipsilateral side was inhibited, whilst type II MVN neurones were unaffected by iontophoetically applied enkephalin. In a recent study, Sulaiman and Dutia (1998) using extracellular and whole-cell patch clamp recording techniques *in vitro,* demonstrated that the large majority of MVN neurones were inhibited by the δ-receptor agonists [D-Ala^2, D-leu^5]-enkephalin (DADLE), and [D-Pen^2 , Pen^5]-enkephalin (DPLPE), the inhibition being antagonised by the selective δ-receptor antagonist naltrindole. Selective μ and κ–opioid receptor agonists failed to elicit responses in any of the MVN cells tested. The effects of DADLE and DPLPE on the MVN cells are likely to be mediated through postsynaptic δ–opiate receptors, as their inhibitory effects persisted after blockade of synaptic transmission.

Substance P

Immunoreactive fibres and terminal endings to substance P have been identified within the vestibular nuclei, particularly in the inferior vestibular nucleus and in the caudal region of the MVN (Nomura *et al.,* 1984). Substance P has been shown to be present in the vestibular nerve (Usami, 1991). As the main transmitter released by these primary afferent fibres is thought to be an excitatory amino acid, it has been suggested that substance P is co-localised with glutamate or aspartate in some of these fibres. Recent *in situ* hybridization studies have demonstrated the presence of MVN neurones immunoreactive to NK-1, the specific receptor for substance P (Maeno *et al.,* 1993). Using intracellular recording techniques Vibert *et al.* (1996) demonstrated that substance P has two distinct actions on MVN cells recorded *in vitro*, 90% of cells were depolarised by bath application of substance P agonists, while the remaining 10% were hyperpolarised. Both responses persisted during synaptic blockade, suggesting that NK-1 receptors are located postsynaptically in the MVN.

Adrenocorticotrophic hormone

Adrenocorticotrophic hormone (ACTH) is a 39-amino acid peptide secreted from the anterior pituitary in response to corticotropin-releasing hormone, whose levels increase during certain physiological conditions, such as a stress. Short fragments of this hormone without corticotrophic activity, $ACTH_{4\text{-}10}$, have been demonstrated to alter the excitability of MVN neurones recorded *in vitro* (Darlington *et al.*, 1990, 1996), the predominant response being a decrease in spontaneous discharge rate. Whether the site of action of $ACTH_{4\text{-}10}$ within the MVN is a specific $ACTH_{4\text{-}10}$ receptor, or some other receptor type which this compound modulates remains unclear at present.

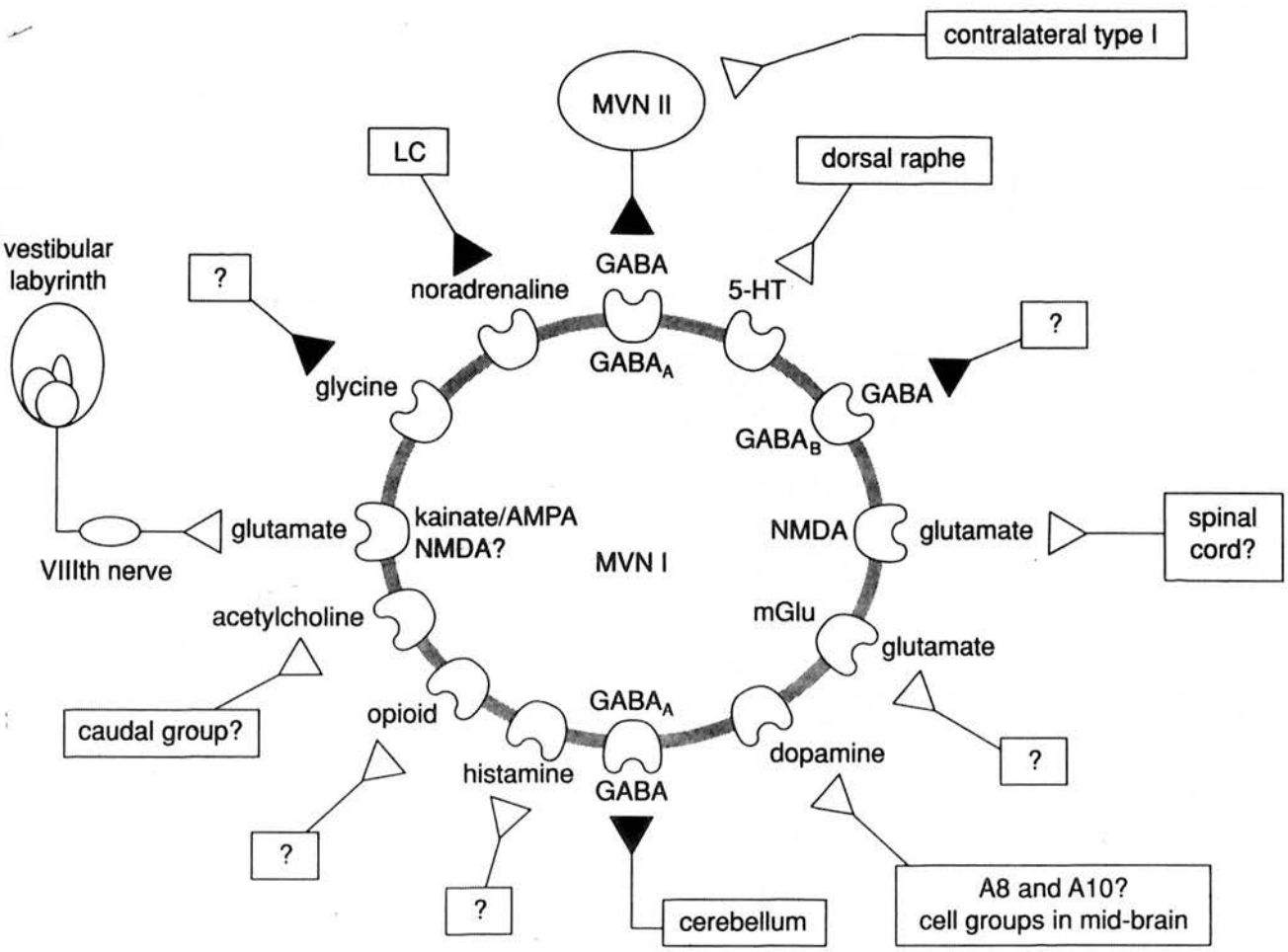


Figure 1.4

Schematic diagram illustrating the transmitters and receptors involved in the synapse between the vestibular nerve and the vestibular nucleus (VN) neurones in the brainstem, and those involved in other synapses with VN neurones. LC locus coeruleus; mGlu, metabotropic glutamate receptors; MVN, medial vestibular nucleus type I neurone. Filled triangles indicate inhibitory actions.

Adapted from Smith and Darlington, (1996).

Summary

The vestibular nuclei constitute a multiple modality sensory integration centre, receiving peripheral information from the vestibular labyrinth as well as afferent inputs from the inferior olive, cerebellum and information from the contralateral vestibular nuclei via the extensive commisural system. Together, these inputs ensure that the appropriate eye movements result as a consequence of a given head movement. It is not surprising therefore, that numerous neurotransmitters are involved in the regulation of central vestibular pathways modulating the sensitivity of postsynaptic receptors to the varying inputs which this area receives. However, the functional roles of the various neuromodulatory systems remain to be characterised.

VESTIBULAR COMPENSATION

Introduction

Vestibular compensation (VC) is the process of behavioural recovery which occurs following the removal of afferent input from the vestibular labyrinth on one side, either by surgical removal of the vestibular receptors (unilateral labyrinthectomy, UL), or by transection of the vestibular nerve (neurectomy) (for recent reviews see Smith and Darlington,1991; Darlington *et al.*, 1992; Curthoys and Halmagyi, 1995: Dieringer, 1995; Vibert *et al.*, 1997). Immediately following UL a severe debilitating syndrome of ocular motor and postural symptoms develop. The behavioural abnormalities following UL can be separated into *static* or *dynamic* symptoms. *Static symptoms* are those present in the absence of head movement, affecting posture and eye movements, whereas *dynamic deficits* are revealed during vestibular stimuli and comprise deficits in the amplitude and timing of the vestibular-ocular and vestibulo-collic reflexes. In most species, static symptoms undergo a remarkable degree of recovery, while compensation of dynamic deficits is generally much slower and often incomplete.

Vestibular compensation is an excellent model of lesion induced plasticity in the adult nervous system for two major reasons:

I. The extent of the peripheral vestibular lesion can be precisely controlled and reproduced.

II. The anatomy, physiology and function of the vestibular system has been well documented and hence the effect of the lesion can be precisely measured and quantified at the behavioural and neural level (Dieringer, 1995; Brandt *et al.*, 1997).

Despite an enormous volume of literature on the behavioural profile of vestibular compensation and the neural correlates of the compensation process in many different species, a clear explanation of the mechanisms of vestibular compensation has proved elusive.

2.1 Static Symptoms

In 1842, Flourens was the first to describe the static oculomotor and postural symptoms of UL and described the progressive disappearance of these symptoms (cited

in, Lacour 1989). Forty years later, Bechtrew deduced that neural rebalancing must be responsible for the disappearance of the static symptoms (cited in Lacour 1989). Bechtrew demonstrated that if a second labyrinthectomy is performed on the remaining intact labyrinth some weeks after the first, the characteristic symptoms of UL reappear. The appearance of these symptoms after the second labyrinthectomy is known as "Bechtrew's phenomenon" and is caused by neural rebalancing of the vestibular nuclei taking place and then being disturbed by the second UL.

1. Spontaneous nystagmus (SN)

In mammals, immediately after UL there is a sustained horizontal ocular nystagmus (SN) with the quick phase being directed away from the lesioned side, and the slow phase directed towards the lesioned side (see Smith and Darlington,1991; Darlington *et al.*, 1992; Curthoys and Halmagyi, 1995; Vibert *et al.*, 1997). SN however, is absent in amphibians (Dieringer, 1995). The time required for the disappearance of SN in light varies according to the species: from hours in the rat and guinea-pig, to a few days in the cat and monkey, to approximately one week in man (Hamann and Lannou, 1988; Shafer and Meyer, 1974; Maoli, 1983, 1985; Curthoys *et al.*, 1988: Fetter and Zee, 1988: Cass, 1992). The recovery of SN is vulnerable during the early phase of VC. Mildly stressful events, such as lifting the animal up, can cause SN and the acute postural symptoms to reappear (Jensen, 1979). Such a relapse is known as "decompensation".

2. Ocular skew deviation

In humans, UL also causes ocular skew deviation so that the eye on the lesioned side moves down in the orbit relative to the eye on the intact side which remains in the normal position (Halmagyi *et al.,* 1979; Curthoys and Halmagyi, 1991). In guinea pigs (Schafer and Meyer, 1974), rabbits (Baarsma and Collewijn, 1975), rats (Sirkin *et al.,* 1984), and frogs (Dieringer and Precht, 1981,1982), there is a comparable deviation in that, the eye on the lesioned side bulges outwards while the eye on the intact side sinks into the orbit.

3. Head tilts

As well as SN, UL causes changes in the maintained posture of the head. In humans after UL there is a modest head tilt, but in other species there are marked changes in maintained head posture. In guinea-pigs, rats, cats, frogs and monkeys, UL causes both a "yaw head" deviation and a "roll head" tilt toward the side of the lesion (MacNaughton and McNally 1946; Sirkin *et al.*, 1984; Xerri 1985; Curthoys *et al.*, 1988; Hamann and Lannou, 1988; Fetter and Zee, 1988; de Waele 1989; Cass, 1991). The recovery of such postural deficits is species specific, taking only a few hours in rats but days in the guinea pig and cat. Putkonen *et al.* (1977) demonstrated that, if cats were kept in the dark, the head remained tilted, with no recovery throughout the duration of the dark period. Re-exposure to light was then followed by a rapid decrease in head-tilt. Furthermore, if compensated animals were put back in the dark they re-acquired a strong head-tilt.

It should be stressed that the rate of recovery of the *static* symptoms described above varies considerably between species. A general recovery of the static symptoms is achieved in approximately 24h in the rat (Llinas *et al.*, 1979; Hamann and Lannou, 1988), 52h in the guinea pig (Smith and Curthoys, 1988a,b; Vibert *et al.*, 1997; Ris *et al.*, 1995,1997), up to 5 weeks in the cat (Lacour *et al.,* 1985; Maoli and Precht, 1985; Maoli *et al.*, 1983), 3 weeks in the monkey (Fetter and Zee 1988), and 2 months in the frog (Dieringer and Precht 1981; Dieringer, 1995).

The type of lesion is important to the timing of the appearance of the symptoms of labyrinthectomy. In the rat, surgical labyrinthectomy produces severe symptoms which occur within a few minutes after recovery from anaesthesia, and which diminish considerably during the next 6 h (Hamann and Lannou, 1988). Studies in which a chemical labyrinthectomy was performed using injection of sodium arsanilate into the middle ear, have reported that the static symptoms are not observed until several hours after the procedure, are at there most severe 24h after UL, and persist for 4 days (Kaufman *et al.*, 1992, 1994). In contrast, following surgical UL in rats, only a weak head tilt is observed at 24h post-UL. Studies have also reported differences in the severity of symptoms between unilateral labyrinthectomy (pre-ganglionic

labyrinthectomy) and unilateral neurectomy (post-ganglionic labyrinthectomy) in mammals. Head tilt, for example, has been reported to be more severe after neurectomy than after labyrinthectomy (Zennou-Azogui *et al.*, 1993). In rats, SN was found to disappear by 4 days post surgery both in animals which had received a labyrinthectomy, and in animals which had received a neurectomy (Sirkin *et al.*, 1984; Li *et al.*,1995). Circular walking was only observed in rats following UL but not after vestibular neurectomy (Li *et al.*,1995). In frogs, however, Kunkel and Dieringer (1994) found no difference in the time taken for postural recovery to occur between pre- or post-ganglionic labyrinthectomy. Li *et al.* (1995) also demonstrated histological differences between pre- and post-ganglionic lesioned animals. After UL, degenerated axons and terminals were distributed throughout all terminal regions of primary vestibular fibres on the lesioned side, while after vestibular neurectomy, the degeneration was more limited.

These results suggest that care must be taken when comparing results between studies where different surgical procedures have been performed.

2.2 Dynamic deficits

Whereas the static symptoms produced by UL and their recovery have been known about for over 150 years, the dynamic deficits associated with the lesion were first described 50 years ago (cited in Curthoys and Halmagyi, 1995).

Studies of the VOR show a permanent VOR gain asymmetry following UL (Baarsma and Collewijn, 1975; Maioli *et al.*, 1983; Maioli and Precht, 1985; Cass and Goshgarian, 1991). For example, in monkeys, immediately after UL the gain of the VOR (i.e. the eye velocity response for a given head rotation) is smaller than in control animals for both ipsilesional and contralesional rotations (Fetter and Zee, 1988). Three months after surgery the gain of the VOR is approximately 60% of the preoperative VOR gain for ipsilesional rotations, whereas a gain of about 80% was recorded for contralesional rotations. The asymmetry of the VOR gain appears to be permanent in all species studied since abnormalities in the gain of the VOR can be detected many months and even years following labyrinthectomy.

2.3 Spontaneous discharge rate of vestibular neurones recorded in the early stages after unilateral labyrinthectomy

In a pioneering study on the decerebrate cat, Precht *et al.* (1966) showed that the appearance of the static symptoms following UL were correlated with the loss of the normal spontaneous resting activity of neurones in the vestibular nuclei ipsilateral to the lesion. In the same study, the authors showed that, compensation of the static symptoms was related to a restoration of the resting activity in these neurones. These results indicated to the authors that the return of resting activity to vestibular nucleus neurones was important for recovery from the static symptoms of UL. This hypothesis has received experimental support from *in vivo* electrophysiological studies of vestibular neurones which have studied the sequences of changes in the resting activity in the VNC at varying times following UL, in several different species (Shimazu and Precht, 1965, 1966; McCabe and Ryu, 1969, 1972; Dieringer and Precht, 1977, 1979a; Markham *et al.*, 1977; Markham and Yagi, 1984: Ried *et al.*, 1984; Hamann and Lannou, 1988; Smith and Curthoys 1988a,b; Newlands and Perachio, 1990; Zennou-Azogui *et al.*, 1993; Ris 1995, 1997).

Type I neurones

Type I neurones are excited by ipsilateral horizontal canal afferents. As a consequence of UL, an immediate reduction in the number of Type I neurones recorded in the ipsilateral MVN was observed (Precht *et al.*, 1966; McCabe and Ryu, 1969, 1972; Smith and Curthoys, 1988b; Hamann and Lannou, 1988; Newlands and Perachio, 1990; Ris *et al.*, 1995). The average resting discharge rate of those neurones recorded in the lesioned nucleus was also very low (Fig 2.1). In the guinea pig MVN ipsilateral to the side of the lesion at 0-8h post-UL the nucleus is almost devoid of type I neuronal activity (Smith and Curthoys, 1988b). In contrast, type I neurones in the contralateral MVN show an increase in their resting discharge rate (Smith and Curthoys, 1988a). During the course of the compensation process, the imbalance in discharge rates of type I neurones between the ipsilateral and contralateral nuclei gradually disappears (fig 2.1). Early studies showed that, in the guinea pig, resting activity had returned to type I neurones in

the ipsilateral nucleus 52h post-UL (Smith and Curthoys, 1988b). More recently, Ris *et al.* (1997) demonstrated that resting activity of type I neurones in the ipsilateral VNC was first observed 12 to 16 h post-UL. In guinea pigs, the static symptoms are well compensated by 52 hours post-UL (Smith and Darlington, 1991), and the return of the resting activity to ipsilateral type I neurones by 52-60h post-UL correlates with the disappearance of the static symptoms. It is unlikely however, that the recovery of resting discharge rates in VN neurones after UL is the only mechanism responsible for behavioural recovery. This conclusion is based on two observations. First, in guinea pigs during the first 45-90 min following UL, when neuronal activity in the ipsilateral VN is very low or absent (Smith and Curthoys, 1988b; Ris *et al.*, 1997), animals are able to stand-up and maintain their balance. Second, during the first 10h post-UL SN diminishes markedly (Ris *et al.*, 1995,1997).

Type II neurones

Type II vestibular neurones are excited by contralateral horizontal canal afferents via the commissural pathway and hence type II neurones in the VNC ipsilateral to the lesion do not lose their main source of excitation following UL. Ipsilateral type II MVN neurones recorded up to 8 h following UL in anaesthetised guinea pigs were encountered more frequently and had an average resting discharge rate which was significantly higher than normal (Smith and Curthoys 1988b). Type II responses were encountered less frequently in the 52-60h group and their resting discharge rate was lower than in the 0-8 h group (Smith and Curthoys, 1988b). In the contralateral nucleus at 0-8h post-UL, type II neurones were reported to be difficult to find and their average resting discharge rate was lower than in control animals (Smith and Curthoys, 1988a)

Studies using [^{14}C]2-deoxyglucose uptake as an indicator for metabolic activity have demonstrated a considerable decrease of deoxyglucose uptake within the lesioned VNC a few hours following UL in the rat (Patrickson *et al.*, 1985; Luyten *et al.*, 1986), which persisted for up-to 20 days post-UL. Similar findings were observed in the frog (Flohr *et al.*, 1981, 1989). These studies support the electrophysiological data that, soon after UL, neuronal activity is absent or significantly decreased in the lesioned VNC.

2.4 Spontaneous discharge rate of vestibular neurones recorded from compensated animals

In guinea-pigs which had been allowed to recover for between 8 and 12 months after UL, type I neurones in the lesioned MVN were found to be firing at a slightly higher rate than animals in the 52h post-UL group, although they had not returned to control levels (Smith and Curthoys, 1988b). In contrast, the activity of type II neurones had recovered to control levels (Smith and Curthoys, 1988b). In the contralateral nucleus, the increase in discharge rate of type I neurones observed in acutely UL animals had recovered to control levels by 52-60h post-UL. Whereas type II MVN neurones showed a slight increase in resting discharge rate at 52-60h, which became significant by 8-12 months post-UL (Smith and Curthoys, 1988a). Although type I neurones in the ipsilateral nucleus of guinea pigs show a significant recovery after 1 week, this does not appear to be the case in cats (Precht *et al.,* 1966; Ried *et al*., 1984; Markham and Yagi, 1984). Type I MVN neurones recorded ipsilaterally 1 month after UL were still found to firing at levels significantly lower than control, whereas in the contralateral nucleus type I responses had recovered to control levels (Ried *et al*.,1984; Markham and Yagi, 1984).

In a recent study, Ris *et al.* (1995), recorded neuronal activity in alert guinea pigs 1 week post-UL. In addition to the ipsilateral MVN, the SVN and LVN were included in their study. This study showed that, following vestibular compensation of the static symptoms, the resting discharge rates of neurones in these three nuclei did not differ from control levels. In contrast, just post-operatively, the resting discharge of neurones in all nuclei were significantly decreased when compared with control groups. Unfortunately, these results reflect the entire population of neurones recorded and, the neurones were not divided into type I and type II subgroups.

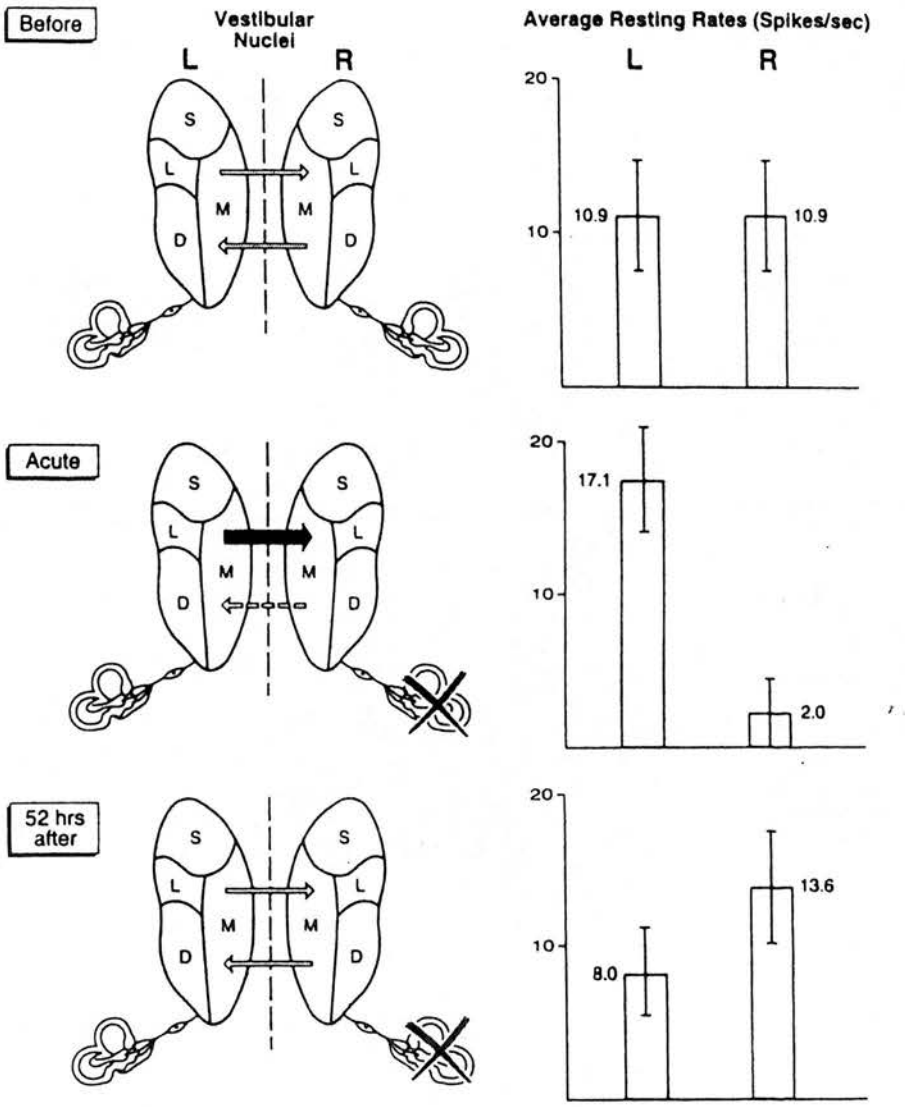

Before
Vestibular Nuclei
L
R
S
L
M
D
Average Resting Rates (Spikes/sec)
20
10
10.9
10.9
Acute
17.1
2.0
52 hrs after
13.6
8.0

Figure 2.1

Diagram to explain how the interaction between the medial vestibular nuclei results in changes in resting activity after UL. The vestibular nuclei are shown schematically, and the adjacent graphs show the average resting rate in each nucleus (as obtained from guinea pigs before and after UL (data from Smith and Curthoys, 1988a,b).

1). Before UL there is an equilibrium condition: Each MVN receives input from the peripheral afferent neurones and exerts inhibition via the commissural fibres on the opposite MVN. The average resting discharge is equal in both nuclei.

2). In the acute stage (0-8h) after UL type I neurones on the lesioned side now receive no afferent input and therefore, the resting discharge rate of cells in the ipsilateral MVN drops to a low value. The reduction in activity of ipsilateral type I neurones releases neurones in the contralateral MVN from tonic inhibition. In turn, the resting discharge rate in the contralateral nucleus increase and they exert a greater inhibition on type I neurones in the ipsilateral MVN (thick arrow)-thus lowering the resting discharge rate of ipsilateral MVN neurones even further.

3). Over the next 52h, cells in the ipsilateral MVN commence firing again and so start to inhibit the neurones in the contralateral MVN. Although the type I neurones in the ipsilateral MVN never fully recover the resting discharge rate seen before UL, the resulting balance between the two groups of VNC is thought to be responsible for the disappearance of the static symptoms.

Adapted from Curthoys and Halmagyi, (1995).

Summary

There is now little disagreement that unilateral destruction of the peripheral vestibular receptors is followed by a fall in the resting activity of VNC neurones on the corresponding side and that this resting activity subsequently recovers. However, there is still some disagreement as to the extent of this recovery, which seems to be species specific. For example, in the cat (Precht *et al.,* 1966, Ried *et al.*, 1984) only a partial recovery of ipsilateral type I neurones has been observed, whereas in the guinea pig, a complete recovery occurs (Smith and Curthoys 1988a,b; Ris *et al.*, 1995,1997). Regardless of whether recovery is complete or not, it is the *return* of resting activity to the once silent VNC which is assumed to be important in bringing about recovery from the behavioural deficits which occur as a result of a UL. Exactly what causes the return of resting activity to the deafferented VNC is unknown although several possible mechanisms have been suggested. To date, there is no clear evidence favoring any existing mechanism and the mechanisms underlying VC still remain a mystery.

2.5 SRUCTURES INVOLVED IN THE PROCESS OF VESTIBULAR COMPENSATION

In order for vestibular compensation to occur structures other than the VNC may be important. These include the cerebellum and inferior olive. In some species, vision also plays an important role in VC.

Role of the cerebellum in VC

A structure intergrating visual and labyrinthine influences to control the VOR pathway, the cerebellum is well placed to receive information about the balance between the two vestibular nuclei through primary vestibular projections via mossy fibres (Precht and Llinas, 1969), and to send an inhibitory output back to the VN through Purkinje cell axons (Angaut and Brodal, 1976).

A large body of evidence suggests that specific areas of the vestibulocerebellum are important in the early stages of vestibular compensation. Haddad *et al.* (1977) showed that in cats which had received a cerebellar lesion involving the flocculus, nodulus and uvula followed by a subsequent unilateral labyrinthectomy three weeks later, compensation of spontaneous nystagmus was significantly delayed. These animals also showed more severe deficits in the timing of the VOR. These initial findings were supported by Schafer and Meyer (1979), who showed that cerebellectomy prior to UL in the guinea pig retarded compensation of nystagmus. Courjon *et al.* (1982a) in the cat, showed more specifically that a unilateral flocculectomy (contralateral to the labyrinthectomy) prior to UL retarded VC. However, when the flocculectomy was performed 16-20 months after a UL the time taken for nystagmus to compensate was not different from animals which had received a labyrinthectomy only. In agreement with Courjon *et al.* (1982a), Igarashi, (1985a) demonstrated that in squirrel monkeys that flocculectomy followed by UL leads to prolonged nystagmus but normal compensation of other behavioural deficits. These studies suggest that the flocculus is essential in restoring normal vestibulo-ocular function after damage to one labyrinth early in the compensation process.

Biochemical studies have shown that Fos, the protein product of the immediate early gene, *c-Fos,* is expressed in the uvula, nodulus, flocculus and ventral paraflocculus

3-24 h following UL (Kaufman *et al.*, 1992; Cirelli *et al.*, 1996). Immediate early genes such as *c-fos* are involved in the first wave of gene transcription following cell stimulation (Hughes and Dragunow, 1995), and their presence suggests that information regarding the imbalance in activity between the two VNC reaches the vestibulocerebellum rapidly after UL.

Recent studies have looked at the activation of intracellular signaling pathways in the cerebellum following UL. Goto *et al.* (1997) demonstrated regionally-selective increases in protein kinase C (PKC) isoforms in rats 6h following UL. More specifically, these changes occurred in the Purkinje cells of the vestibulocerebellum contralateral to the lesion. The increases in PKC immunoreactivity disappeared by 24h post-UL, and no further changes were found at 48h or 8 days post-UL. Nitric oxide (NO) is a diffusible gas, and is well known to act as an intracellular second messenger in the CNS. Kitahara *et al.* (1997) demonstrated that in rats, 12 hours after a UL, NO synthase (NOS) immunoreactivity occurred predominantly in the unipolar brush cells of the ipsilateral flocculus and ventral paraflocculus, with less immunoreactivity seen in the uvula and nodulus. NOS immunoreactivity reached its maximum intensity in these structures 24 h post-UL and gradually disappeared 7 days after surgery. In the second part of this study, rats received continuous infusion of a NOS inhibitor, N^{W} -nitro-L-arginine methyl ester (L-NAME), into the bilateral flocculi two hours before UL. Under these conditions spontaneous nystagmus was found to be significantly higher than in rats which received vehicle infusions and L-NAME treated animals recovered more slowly than animals which received a UL alone (Kitahara et al., 1997). These findings suggest that up-regulation of NO production in ipsilateral floccular unipolar brush cells facilitates vestibular compensation, whereas, in the contralateral floccular Purkinje cells an upregulation of PKC occurs.

Role of the Inferior Olive in VC

Lesions of the inferior olive disrupt VC. Llinas (1975) reported that lesioning the inferior olive in the rat prevented VC from occurring, or caused decompensation in rats which had recovered from a UL six months previously. De'Sperati *et al.* (1993) has

shown that in labyrinthine-intact rats, inactivation of the inferior olive by reversible cooling or chemical lesions, causes an increase in the firing rate of Purkinje cells and consequently a decrease in the resting activity of contralateral MVN neurones. Biochemical studies have reported an increase in the incidence of Fos labelling in the contralateral beta nucleus of the IO 24 h following a chemical UL (Kaufman *et al.*, 1992). In a more recent study looking at earlier time points, Kitahara *et al.* (1995) demonstrated that Fos immunoreactivity was increased in the contralateral beta nucleus 1 h after UL, reaching its maximum intensity by 6h, after which the level of immunoreactivity was gradually reduced. Sato *et al.* (1997) also demonstrated an increase in the expression of Fos immunoreactivity in the contralateral beta nucleus 3 and 6 h post-UL. Taken together, the above results strongly suggest that the beta nucleus of the inferior olive may have a role in vestibular compensation. It is possible that under normal circumstances, the contralateral inferior olive contributes to the resting activity of MVN neurones via the vestibulocerebellum. Thus following UL, the inferior olive nuclei detect the imbalance in resting activity between the vestibular nuclei, both through the direct projection from the MVN to the beta nucleus of the IO (De Zeeuw, 1994), and from abnormal visual stimuli via the dorsal cap. Synaptic projections from the inferior olive to the vestibulocerebellum may help to recalibrate the imbalance in activity between the vestibular nuclei by altering the inhibition exerted by the Purkinje cell inputs onto the MVN.

Summary

Given the multiplicity of sites where lesions impair either the initiation or maintenance of vestibular compensation, it seems likely that many CNS structures are involved in restoring the resting discharge rate of ipsilateral VNC neurones after UL.

2.6 NEUROCHEMICAL CHANGES FOLLOWING VC

Many behavioural studies have examined the effect of various drugs on the time course of vestibular compensation, usually by measuring the disappearance of spontaneous nystagmus or the recovery of postural deficits (Smith and Darlington, 1988,1989; Aoki *et al.*, 1996). However, substances administered systemically may affect VC by indirect means, for example, by affecting blood pressure or alertness. More direct evidence comes from studies which have applied substances directly to the VNC via a cannula (Sansom *et al.*, 1990). A few studies have directly measured the neurochemical changes in the VNC which accompany VC (Thompson *et al.*, 1986; Henley and Igarshi, 1991; Li *et al.*, 1996), and it is only recently that studies have started to use the *in vitro* brainstem slice preparation, to study the effects of compounds on the spontaneous activity of vestibular nucleus neurones following unilateral labyrinthectomy (Smith and Darlington, 1992).

Glutamate

Smith and Darlington (1988), showed that systemically administered CPP, a competitive NMDA receptor antagonist, or the non-competitive antagonist, MK-801 caused a loss of compensation in animals which has received a UL 2-3 days previously, but did not produce decompensation at 12 days, one month or three months post UL. When administered in the first 24h post-UL, these compounds caused a disruption of the compensation of SN (Darlington and Smith, 1989). Systemic administration of MK801 to frogs caused decompensation; again this effect was time-dependent and could not be observed 60 days post-UL (Flohr and Luneberg, 1993). In the rat, i.p. administration of MK-801 also caused decompensation two weeks post-UL, but had no effect after this time (Kitahara *et al.*, 1995, 1996). Studies, in which NMDA receptor antagonists were administered directly into the VNC confirmed the previous findings that injection directly into the lesioned VNC resulted in ocular motor and postural decompensation in guinea-pigs two to three days post-UL (Sansom *et al.*,1990; DeWaele *et al.*, 1990). Curiously, systemic or i.c.v injection of MK-801 before UL had the opposite effect to post-UL injections, in that SN and postural symptoms of UL were reduced (Sansom *et*

al.,1992, 1993a; Aoki *et al.*, 1996).

Evidence from *in vitro* electrophysiological studies, showed that cells recorded from brain slices containing the MVN taken from guinea pigs which had received a UL and subsequently compensated for between 3 days to 2 months showed no differences in either the number of neurones responding to NMDA receptor antagonists or agonists, or an increase in their affinity or efficacy of NMDA receptors compared to control cells (Smith and Darlington, 1992a). To date no studies have looked at compensating MVN neurones 0-3 days post-UL. Thus, NMDA receptors in the ipsilateral MVN do not appear to be involved in the long term maintenance of VC, as suggested from the behavioural studies (Darlington and Smith, 1989; Sansom *et al.*,1990; DeWaele *et al.*, 1990; Smith and Darlington, 1992; Flohr and Luneberg, 1993; Kitahara *et al.*, 1995, 1996).

Results from high performance liquid chromatography (HPLC), immunohistochemical and *in situ* hybridisation studies also suggest that there is no overall increase in the number of NMDA or AMPA receptors in the ipsilateral VNC during the development of compensation, or over the long term. Henley and Igarashi (1991) showed that the levels of glutamate and aspartate were similar in both the ipsilateral and contralateral VNCs 10 months after UL in squirrel monkeys. In the rat, Li *et al.* (1996) recently reported that lower levels of glutamate were found in the MVN ipsilateral to the lesion from 2-30 days post surgery, but this was found only to be significantly lower at 30 days. The changes in aspartate concentrations were similar to those for glutamate. Raymond *et al.* (1989) reported that the number of NMDA binding sites in the ipsilateral VNC did not change during VC in the rat, a finding subsequently confirmed by DeWaele *et al.* (1994). Immunohistochemical studies of AMPA receptors also indicate no regional asymmetries between the ipsilateral and contralateral VNC in the rat between 4 and 30 days post-UL (Li *et al.*, 1995; Rabbath *et al.*, 1996).

In considering the behavioural, electrophysiological and biochemical studies together, it seems clear that NMDA receptors contribute to the early stages of compensation, i.e. when compensation is still developing. It is clear that, whatever NMDA receptors contribute to VC, they do so within a limited time period which varies

between species.

GABA

Li *et al.* (1996) measured GABA levels using HPLC from tissue samples taken from rats at varying time points after UL. GABA concentrations in the VNC showed trends towards early decreases which was bilateral, followed by later recoveries to control or higher levels. The later changes in GABA concentrations were especially prominent in the MVN, where significant increases were found at 7 and 30 days post-lesion. In the squirrel monkey, Thompson *et al.* (1986) demonstrated that GABA immunoreactivity was significantly higher on the lesioned side 3 and 6 days after UL, and Calza *et al.* (1992) observed a decrease in benzodiazepine binding in the lesioned MVN within 3h post-UL suggesting a functional down-regulation of $GABA_A$ receptors.

Acetylcholine

Acetylcholine (ACh) has been implicated in the recovery processes of both the postural and gaze control systems (Kasik *et al.*, 1986). The ACh agonists, eserine and nicotine, slow compensation (Abeln *et al.*,1981; Bienhold and Flhor,1980), whereas the ACh antagonists, atropine and scopolamine, accelerate compensation in the frog. Similar effects on the pattern of VC were observed in the squirrel monkey after administration of ACh agonists and antagonists (Ishikawa and Igarashi, 1985). Acetylcholinesterase (AchE) inhibitors also induce changes in compensation (Bienhold and Flohr, 1980). Torte-Hoba *et al.* (1996) recently investigated the changes in the levels of AchE, in the VNC, at different time points after UL. They showed that at 6 hours post-UL, the vestibular nuclei on the side ipsilateral to the lesion showed a decrease in staining for AchE, whereas contralateral nuclei did not differ from control levels. The reduction of AchE labelling was localised to the rostral part of the VNC. This imbalance of AchE labelling was also observed at 30 h post-UL although it was less consistent than at 6 h post-UL, and inter-individual variations occurred. By 3 weeks and 1 year post-UL, a strong asymmetry still persisted, but was not as consistent.

Ca^{2+} Channel modulators

It has recently been reported that depolarisation of the vestibular nerve causes an increased calcium influx in ipsilateral MVN neurones, measured using rhod 2 fluorescence (Takahashi *et al.*, 1994a). This increased calcium influx was blocked by an NMDA receptor antagonist or reduced by the L-type calcium channel antagonist, nifedipine. Behavioural studies have also looked at the effects of Ca^{2+} channel antagonists on vestibular compensation. In frogs, administration of the T-type Ca^{2+} channel antagonist, flunarizine enhances the compensation of roll head tilt (Takahashi and Akaike, 1991). In the guinea pig, Tolu *et al.* (1988a,b) reported that administration of flunarizine significantly accelerated compensation of the postural and ocular motor symptoms observed following UL, and that SN had disappeared by 24h post-UL. A single injection of the L-type Ca^{2+} channel antagonist, verapamil 1h before UL resulted in an acceleration of SN compensation in the guinea pig, whereas repeated injections in the first 24h following UL had no effect (Darlington and Smith, 1992). Calmidazolium chloride, the Ca^{2+} dependent enzyme inhibitor, administered by intraventricular injection in guinea pigs following UL, caused a dramatic decrease in the amount of SN observed at 10h post-UL. However, this effect was not permanent, in that, by 25h post-UL the frequency of SN had returned to levels observed in untreated animals (Sansom *et al.*, 1993).

How these compound act to alter the time course of VC is not known. To date, there is no direct evidence that intracellular Ca^{2+} levels change in the ipsilateral VNC following UL.

Histamine

Motion sickness in humans arises from a sensory mismatch between visual input and proprioceptive input, treatment of these symptoms is clinically treated with antihistamines, anti-cholinergics or sedatives (Wood, 1979; Lacour, 1998). These compounds presumably act to depress the vestibular response. Betahistine is a partial agonist at the H_1 receptor, and an antagonist at the H_3 autoreceptor (Arrang *et al.*,1983, 1985,1987), and has been demonstrated to facilitate compensation in cats following a

vestibular neurectomy (Tighilet and Lacour, 1995). A recent immunocytochemical study using an antibody against histamine showed a significant decrease in histamine staining in the vestibular and tuberomammilary nuclei bilaterally 1, 3 and 52 weeks after neurectomy (Tighilet and Lacour, 1997). In control animals which had been treated with betahistine, a near-total lack of histamine immunoreactivity in all the vestibular nuclei as well as in the tuberomammillary nuclei was observed (Tighilet and Lacour, 1997). These results suggest that the vestibular imbalance caused by UL results in the activation of histamine neurones in the tuberomammillarly nucleus which project to the VNC. Horri *et al.* (1993) have demonstrated a 200% increase in the amount of histamine released following electrical stimulation of the inner ear. The increase observed in the rate of vestibular compensation following treatment with betahistine may not be due to a direct action on VNC neurones. H_3 receptors have been shown to be co-localised on the axon terminals with dopamine, noradrenaline, and glutamate receptors, suggesting that the effects of betahistine maybe to modulate the levels of other neurotransmitters within the CNS, which in turn act on VNC neurones.

Adrenocorticotrophic hormone (ACTH)

Flohr and Luneberg (1982), reported that the neuropeptide fragment, ACTH-(4-10) was effective in enhancing the compensation of roll head tilt in frogs. Compensation of spontaneous nystagmus was enhanced in the squirrel monkey by treatment with ACTH-(4-10) (Igarashi *et al.*, 1985b). SN was also reduced in the guinea pig following treatment with ACTH-(4-10), whereas compensation of postural deficits was unaffected (Gilchrist *et al.*, 1990). *In vitro* electrophysiological studies have demonstrated that MVN neurones, from both lesioned ipsilateral and intact contralateral nuclei in compensated animals, are similarly inhibited following bath application of ACTH-(4-10) (Darlington *et al.*,1993).

Summary

Many neurotransmitters show changes following UL and this raises the question as to what role this neurochemical rearrangement plays in the process of VC (Lacour, 1988). It is tempting to speculate that these changes in neurotransmitter levels are accompanied by changes in receptor levels, but to date there is little evidence for such changes.

2.7 POSSIBLE MECHANISMS OF VESTIBULAR COMPENSATION

The return of resting activity to the silenced VNC is assumed to be important in bringing about the compensatory process which occurs following UL. Several possible mechanisms have been suggested as to events which may be important in restoring the resting activity of ipsilateral VNC neurones.

1. Reactive Synaptogenesis

Reactive synaptogenesis is the process where healthy axons sprout into the synaptic contacts vacated by the degenerating axons of the neurones which have been cut during surgery. Reactive synaptogenesis has often been suggested as a possible explanation of vestibular compensation. Although there is evidence to support its occurrence in the frog (Dieringer *et al.*, 1984; Will *et al.*, 1988), the evidence from most mammalian species (Korte and Friedrich, 1979; Gacek *et al.*, 1988; Raymond *et al.*, 1991) is that the whole process of degeneration and sprouting develops too slowly to be the primary cause of compensation of the static vestibular symptoms, which occurs within 24h in the rat, 52 h in the guinea pig and 2-5 weeks in the cat. Whilst structural changes thus seem likely to be too slow to explain the initial return of resting activity to the deafferented nucleus, they may underlie the long-term stability of vestibular compensation (Gacek *et al.*, 1988; Curthoys and Halmagyi, 1995).

2. Denervation supersensitivity

It is possible that neurones in the vestibular nuclear complex, which have been deprived of a massive amount of peripheral input by the vestibular lesion, become more sensitive to the transmitter normally released by the now silent primary afferents. Other afferent inputs synapsing with VNC neurones probably also release the same transmitter as the now silent vestibular nerve (Curthoys and Halmagyi, 1995). Several studies have suggested that the return of the resting activity to silenced cells may be due to an increase in the affinity, efficacy or number of NMDA receptors on the ipsilateral VNC neurones (Smith and Darlington, 1988; Darlington and Smith, 1989; De Waele *et al.*, 1990; Sansom *et al.*, 1990; Pettorossi *et al.*, 1992; Flohr and Luneberg, 1993.).

However, electrophysiological (Knopfel and Dieringer, 1988; Smith and Darlington, 1992), pharmacological (Raymond *et al.*, 1989), and biochemical studies (De Waele *et al.*, 1994), do not support the idea that there is an increase in the number or sensitivity of NMDA receptors in the ipsilateral neurones. Therefore it is unlikely that excitatory amino acids acting on NMDA receptors causes the gradual return of resting activity to the lesioned neurones.

3. Change in efficacy of vestibular commissures

Vestibular compensation in the frog is associated with an increase in efficacy of excitatory brainstem commissural input to lesioned vestibular neurones (Kunkel and Dieringer 1994; Dieringer and Precht, 1977, 1979; Dieringer, 1995). However, studies in mammalian species have failed to find any corresponding change in commissural efficacy (Precht *et al.*, 1966; Smith *et al.*,1986b; Smith and Curthoys, 1988a; Newlands and Perachio, 1990a,b). The failure to find any corresponding change in commissural efficacy can most easily be explained by the fact that in mammalian species, the vestibular commissures are functionally inhibitory between type II MVN neurones (see Galiana *et al.*, 1984 for a discussion).

4. Role of non-vestibular inputs

Other studies have suggested that non-vestibular sensory inputs play an important role in the early stages of VC. The cerebellum and inferior olive appear to be essential for VC during the early stages (Haddad *et al.*, 1977; Courjon *et al.*, 1982a; Llinas, 1975), as does vision (Igarashi, 1981). According to the *cerebellar shutdown hypothesis*, originally proposed by McCabe and Ryu (1969), the cerebellum reduces the activity in the vestibular nuclei by modulating its inhibitory output to these brainstem areas, thus rebalancing the activity between the two VNC. Both electrophysiological (Haddad *et al.*, 1977; Schafer and Meyer, 1974; Courjon *et al.*, 1982a; Igarashi and Ishihkawa, 1985a), and biochemical studies (Kaufman *et al.*, 1992; Cirelli *et al.*, 1996; Goto *et al.*, 1997; Kitahara *et al.*, 1997), support the importance of the vestibulocerebellum in VC but, as yet, there is no experimental evidence to suggest that the cerebellar shutdown hypothesis

is correct.

Several groups of studies have shown the importance of spinal afferent input to VC, cutting cervical dorsal roots causes decompensation in squirrel monkeys (Igarashi *et al.*, 1969), and spinal transection causes decompensation in guinea pigs (Azzena 1969, Jensen 1979), and frogs (Fhlor 1982). Azzena (1969) also demonstrated that if the spinal transection was performed before labyrinthectomy, compensation of head deviation was retarded.

To date, experimental evidence does not clearly support any of these hypotheses as a universal explanation of vestibular compensation in all species, but it is probable that all of these mechanisms operate to different extents and at different times during VC. Due to the lack of evidence for any of the above mechanisms, research recently turned to the intrinsic properties of VNC neurones. Recently, Darlington and Smith (1996) suggested a new hypothesis, that the recovery of resting activity in lesioned VNC neurones depends upon the intrinsic properties of these neurones (Gallagher *et al.*, 1985; Serafin *et al.*, 1991; Johnston *et al.*, 1994). The hypothesis suggests that the immediate silencing of ipsilateral MVN neurones after UL is due to 'neural shock' induced by an elevation of calcium levels in these cells. Although the authors have not been forthcoming with the details of the mechanism of the hypothesis, it is presumed that high levels of glutamate are released from the damaged nerve, subsequently leading to increased depolarisation and the silencing of MVN cells. However, in the study by Li *et al.* (1996) the level of glutamate was shown to be unchanged 1 day post-UL and significantly decreased on the lesioned side 2-30 days following surgery. Unfortunately, there is no direct evidence to support this theory as calcium levels have not been directly measured in deafferented MVN nuclei.

Summary

The remarkable plasticity of the vestibular system is expressed by its ability to adapt in the presence of sensory conflict, i.e. motion sickness in labyrinth intact subjects and the process of vestibular compensation following the loss of one vestibular afferent input. However, in each of these cases, different sets of distributed mechanisms of adaptation may be activated and probably to a different degree in different species (Dieringer, 1995; Brandt *et al.*, 1997). The available literature describes many differences between the species as to the importance of other CNS structures in VC, and to the effects of various compounds on the rate of recovery of the static symptoms. It may be naïve then, to think of the mechanisms underlying vestibular compensation as a single process common to every species; what is more likely, is that vestibular compensation is made up of a number of processes that are involved at different times after UL, reflecting different underlying mechanisms. It is unclear whether the mechanisms of the regeneration of resting activity in the deafferented vestibular nuclei have a presynaptic or postsynaptic locus, or as is more likely, both. Presynaptic changes, such as substitution of non-vestibular sensory inputs and reactive synaptogenesis, have been suggested, as have postsynaptic changes such as upregulation of excitatory postsynaptic receptors (Smith and Curthoys, 1989; Darlington *et al.*, 1992). The return of resting discharge rate to the deafferented VNC neurones following UL could result then, from intrinsic changes within the deafferented vestibular neurones, or from a reorganization of the activity of several regions of the central nervous system, or both. It is also likely that different mechanisms are involved in the initiation of compensation compared to those that maintain it (Curthoys and Halmagyi, 1995; Ris *et al.*, 1997).

CHANGES IN THE EXCITABILITY AND A DECREASED RESPONSIVENESS TO GABA OF RAT MEDIAL VESTIBULAR NUCLEUS NEURONES *IN VITRO* FOLLOWING UNILATERAL LABYRINTHECTOMY.

3.1 INTRODUCTION

Numerous studies employing *in vivo* extracellular recording techniques from medial vestibular nucleus neurones (MVN), have shown that immediately after unilateral labyrinthectomy (UL), the normally high resting activity of (Type I) MVN cells ipsilateral to the lesion is largely abolished (Shimazu and Precht, 1965,1966; McCabe and Ryu, 1969, 1972; Dieringer and Precht, 1977, 1979; Markham *et al.*, 1977; Markham and Yagi, 1984: Reid *et al.*, 1984; Hamann and Lannou, 1988; Smith and Curthoys 1988a,b; Newlands and Perachio, 1990; Zennou-Azogui *et al.*, 1993; Ris *et al.*, 1995,1997). The silencing of MVN neurones is presumed to be due, not only to the loss of excitatory drive from the lesioned vestibular afferents but also to an enhanced commissural inhibition from contralateral MVN cells, which become hyperactive due to the decrease in inhibitory drive from the lesioned side (Smith and Curthoys, 1988b). This marked imbalance in the excitability of MVN cells is thought to cause the severe postural and oculomotor disturbances which immediately follow UL as MVN neurones project directly to the motoneurones innervating extraocular and neck muscles (Fig.1.1). In guinea pigs, during the first 52 hours following UL the static oculomotor and postural symptoms subside (see section 2.1 for a discussion) and this has been shown to be temporally correlated with the restoration of the resting discharge in ipsilateral Type I MVN neurones. The return of resting activity to ipsilateral type I neurones is accompanied by a decrease in the hyperactivity of the contralateral cells, which lessens the imbalance in excitability between the MVNs of the two sides (Smith and Curthoys 1989; Smith and Darlington,1991; Darlington *et al.*, 1992; Curthoys and Halmagyi, 1995: Dieringer, 1995; Vibert *et al.*, 1997;), (Fig 2.1). The cellular mechanisms responsible for the initial rapid restoration of the resting discharge of MVN cells in the lesioned nucleus are not known.

Gallagher *et al.* (1985) were the first to demonstrate using extracellular and intracellular recording techniques that rat MVN neurones fired spontaneous action potentials in a brain slice preparation of the rostral medulla. Over the past 10 years,

with the further development of *in vitro* brain slice preparations containing the vestibular nuclei, it has become clear that MVN neurones posses intrinsic pacemaker-like membrane conductances which generate a regular spontaneous tonic discharge *in vitro* (Darlington *et al.*, 1989; Doi *et al.*, 1990; Serafin *et al.*, 1991a.b; Johnston *et al.*,1994; refer to Gallagher *et al.*, 1992; de Waele *et al.*, 1995, for a review). It may not be the case that the intrinsic properties account for all of the resting activity of vestibular neurones which is observed *in vivo*. It is likely that synaptic inputs from the vestibular nerve and other CNS afferents make a significant contribution. While these intrinsic membrane properties are likely to be important in normal vestibular function, it is also possible that compensatory changes in the intrinsic excitability of these cells may occur during VC. **Part 1** of this study was designed to determine if changes in the excitability of tonically active cells in the lesioned MVN occurs at specific time points following unilateral labyrinthectomy. This aim was achieved by using extracellular single unit recording techniques in order to sample a large population of MVN neurones in slices prepared from animals after UL.

GABA has long been implicated in the control of vestibular reflex function (Curtis *et al.*, 1970; Obata *et al.*, 1967; Precht *et al.*, 1973a,b). Immunohistochemical studies have demonstrated an extensive distribution of GABA-containing cells and processes in the vestibular nuclei of several species (Nomura *et al.*, 1984; Kumoi *et al.*, 1987; Walberg *et al.*, 1990). *In situ* hybridization studies have shown that vestibular neurones express high amounts of $GABA_A$ and glycinergic receptors while both presynaptic and postsynaptic $GABA_B$ receptors were demonstrated in the MVN by immunocytochemical techniques (Holstein *et al.*,1992). The role which GABA may play in vestibular compensation has recently been investigated. Calza *et al.* (1992) observed a decrease in benzodiazapine binding in the ipsilateral MVN within 3h post-UL, and an increase in the level of GABA detected in the VNC 3-6 days following UL have been demonstrated by Thompson *et al.* (1986). **Part 2** of this study tested the hypothesis that there are changes in the responsiveness of MVN neurones in the lesioned nucleus to GABA following UL, by bath application of the

$GABA_A$ agonist muscimol, and the $GABA_B$ agonists baclofen to slices containing the MVN prepared from animals post-UL.

3.2 METHODS

Animals

Male Sprague-Dawley rats, body weight 60-150g (Bantin & Kingman, U.K) were used throughout this work. They were housed in litter groups under controlled conditions:- ambient temperature 21-23 °C, 12h light/12h dark cycle, with free access to food (standard breeder diet; Bantin & Kingman, U.K.) and water.

Anaesthesia

1. Avertin (tribromoethanol)

Avertin (Dyer *et al.*, 1981), anaesthetic was made up fresh at the beginning of every week, according to the following recipe: 2.5g tribromoethanol (,Sigma, UK) was added to 10ml of ethanol (Sigma, UK) and mixed until the tribromoethanol dissolved completely. To this solution, 1.5mls of 2-methylbutan-2-ol (Sigma, UK) was added and subsequently made up to a final volume of 125mls by the addition of normal saline (9g/l), the anaesthetic was stored at 4°C. The solution was injected intraperitoneally (i.p.) at a dose of 1ml/100g body weight (BW) and provided surgical anaesthesia for 30-40min.

2. Halothane

Prior to decapitation, animals were anaesthetised with halothane (Fluothane, May and Baker Ltd., UK) by inhalation. The anaesthetic was administered by placing the animals in a small Perspex box containing a piece of halothane-soaked cotton wool. A sufficient level of anaesthesia was determined by a lack of reflex response to a paw pinch.

Surgery

Male Sprague-Dawley rats (60-130g) were anaesthetised with Avertin anaesthesia. A sufficient level of surgical anaesthesia was determined by a lack of reflex to a paw pinch. Animals were then shaved (Wella, Expert Clippers) in the

area which surgery would involve and the area cleaned with disinfectant wipes (Medichem International UK). Under a microscope (Wild ms-c, Heerburg, Switzerland) a 4cm incision was made above the left ear with a sterile scalpel and the connecting tissue was cut using sharp surgical scissors, the middle ear was opened and the ossicles were removed. The muscles attached to the lambodial ridge were cut close to the bone and reflected to expose the temporal bone. The bony duct of the horizontal semicircular canal was exposed by drilling with a dental drill (Precision P.C.B drill, RS Components) to the point of exit of the VIIth cranial nerve. The area was kept clear with a needle attached to a suction pump (Eschmann VP25, International Market Supply). The horizontal duct was followed anteriorly and opened near its ampullary swelling. Drilling was continued in the plane of the horizontal canal to follow the open duct into the vestibule of the inner ear. The ampulla of the anterior canal was drilled through, and the contents of the vestibular cavity were rinsed with 100% ethanol (Sigma U.K.), which was then aspirated. When bleeding had stopped, the wound was sutured and animals returned to their home cage to recover. In sham operated animals, an identical procedure was used except that the horizontal canal was not opened, and no damage was inflicted on the inner ear.

Behavioural measurements.

Each animal was observed following recovery from anaesthesia, which was complete within 50-60 min after induction. Unilaterally labyrinthectomised animals showed characteristic symptoms including tonic eye deviation, spontaneous ocular nystagmus, circular walking and head deviation toward the side of the lesion, extensor weakness in the ipsilateral limbs, and barrel rolling. These symptoms were absent in sham-operated animals.

Quantitative analysis of circular walking as an index of behavioural recovery

In order to quantify the process of vestibular compensation the occurrence of circular walking was measured. Episodic circular walking has been described

previously in the cat (Putkonen *et al.*, 1977), and the rat (Hamann and Lannou, 1988), and is described as tight circling movements towards the lesion side. Labyrinthectomised animals were placed in an open container for the first time, approximately 2h after the completion of surgery, they were given 1 min to adjust to this new environment and then the number of circles towards the lesion side in the next 3 min period was recorded. This procedure was repeated three times and the mean number of circles ± s.e.m. for that time point was calculated. The whole procedure was repeated at the same time every hour for the next 6 hours. Comparison of circular walking over time was done using a 1 way repeated measures analysis of variance (1 way RM ANOVA) statistical test.

Preparation and maintenance of slice containing MVN in vitro

Once anaesthetised, the animal was decapitated using a small animal guillotine. The head was cleared of pelage and skin and a rapid craniotomy performed. With fine bone ronguers the occipital bone was removed, thus exposing the cerebellum. The parietal and frontal bone were divided by a cut in the midline and the cranium was lifted away from the brain using carefully inserted blunt scissors. The intact brain was freed from the skull by cutting the cranial nerves and membranous tissue with a fine scalpel and tipped into cold (4°C) artificial cerebrospinal fluid (aCSF) (see solutions) bubbled with a gas mixture of 95%O_2 and 5%CO_2. The brain was then placed on a cold dissecting stage and the brainstem extending caudally from the inferior colliculus with the cerebellum still attached cut from the cerebrum. The brainstem containing the overlying cerebellum was laid on its rostral surface and the cerebellum removed by gently lifting it clear of the brainstem with the blunt edge of a scalpel blade and cutting through the cerebral peduncles thereby revealing the MVN sitting prominently on the dorsal surface of the medulla. This remaining block of tissue containing the MVN was cleared of adherent pia and trimmed by making a transverse cut at the level of the obex. The block was then cemented with the floor of the fourth ventricle uppermost to the advancing stage of a Vibroslice (Campden Instruments Ltd, UK). The stage was secured into the chamber of the

Vibroslice and submerged in cold (4°C) aCSF pre-bubbled with a mixture of 95%O_2 and 5%CO_2. The block of tissue containing the MVN was cut in the horizontal plane approximately parallel to the floor of the fourth ventricle, thus, obtaining a slice which contained almost the entire rostro-caudal length of the MVN. The first slice containg the top 100-200μM of the nuclei was discarded. The next slice was cut to a thickness of 450μM and was the thickness of slice used for all experiments. The slices containing the MVN were floated onto a moistened fine hair paintbrush and transferred to an interface-type chamber which was continuously perfused with aCSF and gassed with a mixture of 95%O_2 and 5%O_2, and maintained at a temperature of 32 °C +/- 0.2 °C. The slice lay on a fine mesh in the recording chamber with its undersurface in contact with aCSF delivered to and removed from the chamber at a rate of 1.5mls/min by means of a 4-channel peristaltic pump (Gilson, France). A continuous stream of 95%O_2 and 5%CO_2 gas saturated with water vapour was passed over the slice to provide the tissue with oxygen and keep it moist. Slices were incubated for at least 1 hour in the chamber prior to recording.

Solutions

1. Artificial cerebrospinal fluid (aCSF)

The normal perfusion medium used throughout this project was made up fresh each morning in accordance to the recipe for the standard medium outlined in Llinas and Sugimori (1980), and contained the following (in mM); Sodium chloride 124; Potassium chloride 5; Potassium Dihydrogen orthophosphate 1.2; Magnesium sulphate heptahydrate 1.3; Calcium chloride 2-hydrate 2.4; Sodium hydrogen carbonate26; D-(+) Glucose 10. The aCSF was bubbled with a gas mixture of 95%O_2 and 5%CO_2 to oxygenate it and maintain its pH at 7.4.

2. Muscimol (5-aminomethyl-3-hydroxyisoxazole) and Baclofen (4-amino-3[4-chlorophenyl]butanoic acid)

Muscimol, the $GABA_A$ agonist and Baclofen, the $GABA_B$ agonist were obtained from Sigma, U.K. Small aliquots of stock solution were made in distilled water and

frozen until required. The final concentration was made up in aCSF immediately before use, and applied to the slices by switching the perfusion inlet tube to the appropriate reservoir by means of a three-way tap.

Data collection and analysis

All data collection and analysis programmes were written by Dr. MB Dutia on CED Spike 2 software (Cambridge Electronic Design, UK) and generated by an IBM-compatible microcomputer (DCS 386, UK) linked to a 1401 A/D interface. All data analysis was completed off-line, except when measuring extracellular firing rates which was done on-line.

Conventional glass microelectrodes filled with 2M Na gluconate (resistance 10-12 MΩ) coupled to an Axoclamp 2A amplifier (Axon Instruments, USA) were used to systematically explore the MVN for tonically firing neurones. A microcomputer and 1401 *plus* interface (Cambridge Electronic Design, Cambridge, UK) were used to display the instantaneous discharge frequency, autocorrelation and interspike interval histogram in real time, in order to ensure stable recording conditions. Several tonically active cells were often recorded in the course of a single track, and the location of each track was noted with reference to the boundaries of the MVN. For the purposes of analysis the MVN was divided by eye into three regions (rostral third, middle third and caudal third (Johnston and Dutia,1996), and the mean tonic discharge rate of the MVN cells sampled in each of these regions was calculated. Comparisons of the mean discharge rates of MVN cells in different groups were carried out using the Mann-Whitney ranked sum test (MWRST), and significance was assumed when $p<0.05$. Values are given as mean ± s.e.m. throughout.

The inhibition of the tonic discharge of the MVN cells in response to 60-second test pulse of the agonists were determined and expressed as a percentage of the resting discharge rate for each cell (Dutia *et al.*, 1994). The mean inhibitory effects of each concentration of the two agonists were calculated for all the cells tested, and

logistic functions of the form y=Max*(p/(p+EC50)), were fitted using a least-squares method to the dose-response relationship. Although values were assigned to 100% inhibition for all the responses where the tonic activity of the cells were completely inhibited by the GABA agonists, the parameters of the curve-fitting algorithm were not constrained to within this range. The dose-response relationships were compared using two-way ANOVA, or the Wilcoxon Signed Rank Sum test as appropriate, and significance was assumed when $p<0.05$.

3.3 RESULTS

Behavioural observations following unilateral labyrinthectomy

All animals recovered from anaesthesia within 60 min after induction. Following recovery the animals were observed for the characteristic symptoms of UL. All animals included in the study exhibited the classic symptoms of UL, these included barrel rolling and circular walking towards the lesioned side during the very early stages following recovery from anaesthesia (see fig. 3.3). These symptoms were not observed in rats in the 24, 48h or 1, and 2 week groups. Animals also showed tonic eye deviation, spontaneous nystagmus and ataxia. These symptoms gradually abated as vestibular compensation occurred. Animals in the sham-operated group displayed none of the above symptoms.

In order to quantify the recovery following unilateral labyrinthectomy, the incidence of circular walking was measured over the first 7h following UL. As can be seen from figure 3.3, animals showed a high incidence of circular walking towards the lesioned side during the 2-5 hours post-UL, followed by a significant decrease at 6h and 7h post-UL ($P<0.001$ one-way RM ANOVA).

PART 1 Changes in the excitability of MVN neurones *in vitro* following unilateral labyrinthectomy

Spontaneous discharge rate of MVN neurones in control and sham-operated animals

Extracellular recordings from tonically active MVN cells were made in brainstem slices prepared from normal animals and animals which had received a sham-operation. All MVN cells included in the study showed a regular sustained discharge for 90s. Each MVN was divided into three approximately equal parts, namely rostral, middle and caudal and the rostro-caudal location of each cell was recorded. Previous studies (Dutia and Johnston, 1998), have shown that tonically active cells in the rostral third of the MVN on average, have higher discharge rates than cells located in the middle or caudal thirds of the nucleus. The rostral region of the MVN

has been shown to receive a particularly dense innervation of primary afferents from the ipsilateral horizontal semicircular canal (Stein and Carpenter, 1967: Gacek, 1969; Korte and Friedrich, 1979; Carleton and Carpenter, 1984; Sato *et al.*, 1989). The mean firing rates of MVN cells located in the rostral and caudal thirds of sham-operated and control animals were then compared with the mean firing rates of MVN neurones from the corresponding regions in slices taken from labyrinthectomised rats. (Table 3.1a,b)

Spontaneously active MVN cells were found in all slices prepared from normal, sham-operated and previously labyrinthectomised animals. Cells recorded from the rostral third of the MVN taken from normal animals had a mean resting discharge rate of 15.15 ± 1.1 spikes/s (n=62 cells), those in the middle third, 13.61 ± 1.17 spikes/s (n=59 cells), and cells recorded from the caudal third displayed a mean discharge rate of 13.6 ± 1.27 spikes/s (n=49 cells). There were no differences in the mean firing rates of MVN cells recorded in the left and right nuclei of sham operated animals and these data were therefore combined. In sham-operated nuclei cells in the rostral third were found to be firing at 15.20 ± 1.4 spikes/s (n=21 cells), middle 12.3 ± 1.7 spikes/s (n=21 cells), and those recorded from the caudal third at 11.17 ± 1.5 spikes/s (n=20 cells). As there were no differences between the mean firing rates of control and sham-operated groups, these data were pooled. When pooled, cells in the rostral third of the control group were firing at 15.16 ± 0.9 spikes/s (n=83 cells), middle 13.26 ± 0.98 spikes/s (n=80 cells) and cells in the caudal third 12.3 ± 1.03 spikes/ s (n=66 cells) (Figure 3.1 and Table 3.1a).

Increase in mean discharge rate of MVN neurones recorded from slices prepared from animals 2h-2 weeks following UL

Time-dependent changes were observed in the discharge rates of MVN neurones in the lesioned MVN following UL (Figure 3.1). In slices prepared from animals 2h after UL, the mean spontaneous discharge rate of ipsilateral rostral (14.04 ± 1.18 spikes/s, n= 39 cells), middle (12.52 ± 1.7 spikes/s, n=18 cells), or caudal MVN cells (13.88 ± 1.7 spikes/s, n=28 cells), were not different from controls, even though at

this time-point the behavioural symptoms of unilateral vestibular deafferentation were at their most intense. In contrast, in MVN slices prepared from animals 4 h following UL MVN neurones in the rostral third of the lesioned nucleus had a significantly higher mean spontaneous discharge rate (21.56 ± 1.7 spikes/s, n=32 cells), than rostral cells in the control group (15.16 ± 0.9 spikes/s, n=83 cells, p=0.001, MWRST), Figure 3.1. The mean discharge rate of MVN cells in the middle (14.9 ± 1.7 spikes/s, n=23 cells), and caudal thirds (13.3 ± 1.6 spikes/s, n=25 cells), of the lesioned nucleus were not different from their counterparts in the control group, middle (13.26 ±0.98 spikes/s, n=80 cells), caudal (12.3 ± 1.03 spikes/ s,n=66 cells). The significantly higher discharge rate of rostral MVN neurones in the lesioned nucleus seen at 4hs post-UL was also seen at the 6h time point (18.98 ± 1.66 spikes/s, n=43 cells, $p<0.05$ MWRST), the 24h time point (19.86 ± 1.95 spikes/s, n=36 cells, $p<0.05$ MWRST), the 48h time point (18.62 ± 1.52 spikes/s, n=42 cells, $p<0.05$ MWRST), and at 1 week (19.20 ± 1.7 spikes/s, n=57 cells, $p<0.05$ MWRST), following UL. By the second week post-UL the mean firing rate of cells in the rostral third of the lesioned nucleus cells (16.89 ± 1.36 spikes/s, n=44 cells. Figure 3.1) was not significantly differently from control cells (15.16 ± 0.9 spikes/s, n=83 cells). Table 3.1b.

Cells in the rostral third of the lesioned MVN were also found to be firing at a significantly higher discharge rate than rostral MVN cells recorded from the contralateral nucleus of animals which had received a left-UL at the 6h time point (13.59 ±1.2 spikes/s, n=35 cells, $p<0.05$ MWRST), the 24h time point (13.87 ± 1.69 spikes/s, n=21 cells, $p<0.05$ MWRST), the 48h time point (14.4 ± 1.29 spikes/s, n=40 cells, $p<0.05$ MWRST) and at 1 week post UL(14.54 ± 1.77 spikes/s, n=43 cells, $p<0.05$ MWRST). (Figure 3.1 and table 3.1b).

In slices prepared from animals that had previously undergone left unilateral labyrinthectomy, there were no statistically significant changes in the mean tonic

discharge rates of MVN cells in any region of the nucleus contralateral to the lesion over the 2 weeks after UL (Figure 3.2 and table 3.1b).

In previous *in vivo* electrophysiological studies, the majority of cells have been shown to be Type I and it is these cells which have been found to be silenced after UL, type II neurones receive commissural excitation *in vivo* and are therefore not silenced *in vivo* after UL (Smith *et al* ., 1988b; Ris *et al*., 1995,1997). In the present experiments no differences were found in the number of cells encountered in a single track between control and experimental groups (Figure 3.4). This excludes the possibility that the tonically active cells recorded in post-UL slices were in fact the minority of cells (Type II neurones), as this would have been observed as a marked decrease in the overall number of tonically active cells encountered in the post-UL slices. Analysis of the coefficients of variation of the inter-spike intervals showed that these ranged from 0.04 - 0.12, reflecting the steady regular spontaneous activity of MVN neurones *in vitro*, and revealed no significant changes on either the ipsilateral or contralateral sides after UL (Figures 3.5, 3.6).

Increase in mean discharge rate of MVN neurones recorded from slices prepared from bilaterally labyrinthectomised animals 4h post surgery.

To confirm that the increase in the mean resting discharge rate which was observed in rostral ipsilateral MVN neurones was not simply due an injury of the afferent axons, one group of five animals received a bilateral labyrinthectomy. Both labyrinths were destroyed during the same surgical procedure under Avertin anaesthesia. Behaviourally these animals showed different symptoms to those animals which had received a unilateral labyrinthectomy. There was no sign of spontaneous nystagmus. barrel rolling, or head tilts. In contrast these animals exhibited uncontrolled bobbing up and down head movements and splayed a wide stance. All animals were sacrificed 4h post-lesion and no behavioural measurements were made.

There were no differences in the mean firing rates of MVN cells in the rostral or caudal thirds of both the left and right nuclei (rostral cells; left MVN 15.5 ± 1.35 spikes/s, n=42 cells, right MVN 13.76 ± 1.49 spikes/s, n=30 cells, caudal cells; left MVN 21.18 ± 1.48 spikes/s, n=48 cells, right MVN 22.60 ± 1.87 spikes/s, n=34 cells) and these data where therefore pooled. In the rostral third of both nuclei (14.79 ± 0.99 spikes/s, n=72 cells) the firing rates were not firing differently from those in control animals (15.16 ± 0.9 spikes/s, n=83 cells). However, the discharge rate of MVN cells in the caudal third of both nuclei was significantly higher (21.77 ± 1.16 spikes/sec n=82 cells $p<0.0001$ MWRST), compared to those from control cells (12.9 ± 1.03 spikes/sec n=66 cells), and cells recorded in the rostral third (14.79 ± 0.99 spikes/s, n=72 cells $p<0.0001$ MWRST), (Figure 3.7).

Summary

These results demonstrated that there was a significant increase in the mean resting discharge rate of MVN neurones in the rostral third of the lesioned nucleus following UL, which persisted for 1 week. However, after bilateral labyrinthectomy the increase in excitability of the rostral cells was not seen. Instead, there was a bilateral increase in intrinsic excitability of the caudal MVN cells, perhaps related to the symptoms of vertical movements after the lesion.

Treatment		**Left and Right MVN Spikes/s**
a) Control	rostral	15.15 ± 1.1
	middle	13.61 ± 1.17
	caudal	13.6 ± 1.27
b) Sham	rostral	15.20 ± 1.4
	middle	12.31 ± 1.7
	caudal	11.17 ± 1.5
c) Control and sham combined		
	rostral	15.16 ± 0.9
	middle	13.26 ± 0.98
	caudal	12.31 ± 1.03

Table 3.1a

The mean resting discharge rates of MVN neurones recorded *in vitro* from a) control animals and b) animals which had received a sham operation. As there were no significant differences between these groups, c) the data was pooled.

Treatment		**Ipsilateral MVN spikes/s**	**Contralateral MVN spikes/s**
2h post-UL	rostral	14.04 ± 1.18	13.88 ± 1.7
	middle	12.52 ± 1.7	15.24 ± 1.89
	caudal	14.48 ± 1.7	16.17 ± 2.09
4h post UL	rostral	**21.56 ± 1.7**	17.03 ± 1.7
	middle	14.9 ± 1.79	15.08 ± 1.79
	caudal	13.3 ± 1.6	16.31 ± 14
6h post-UL	rostral	**18.97 ± 1.66**	13.59 ± 1.2
	middle	14.29 ± 1.5	14.04 ± 1.47
	caudal	16.98 ± 2.2	12.65 ± 1.35
24h post-UL	rostral	**19.85 ± 1.95**	13.87 ± 1.69
	middle	15.66 ± 1.64	16.74 ± 1.72
	caudal	12.35 ± 1.7	12.30 ± 1.41
48h post-UL	rostral	**18.62 ± 1.52**	14.4 ± 1.29
	middle	14.24 ± 1.56	12.3 ± 1.7
	caudal	13.0 ± 1.47	11.6 ± 1.46
1 week post-UL	rostral	**19.20 ± 1.7**	14.54 ± 1.77
	middle	14.59 ± 1.6	13.27 ± 1.8
	caudal	15.01 ± 2.32	12.95 ± 1.7
2 weeks post-UL	rostral	17.02 ± 1.34	16.4 ± 1.4
	caudal	14.05 ± 1.27	14.19 ± 1.6

Table 3.1b

The mean resting discharge rates of MVN neurones *in vitro* recorded from slices prepared from animals which had previously received a left labyrinthectomy at the time stated. Ipsilateral MVN = ipsilateral to lesion. Values in bold indicate significantly different when compared to corresponding control region.

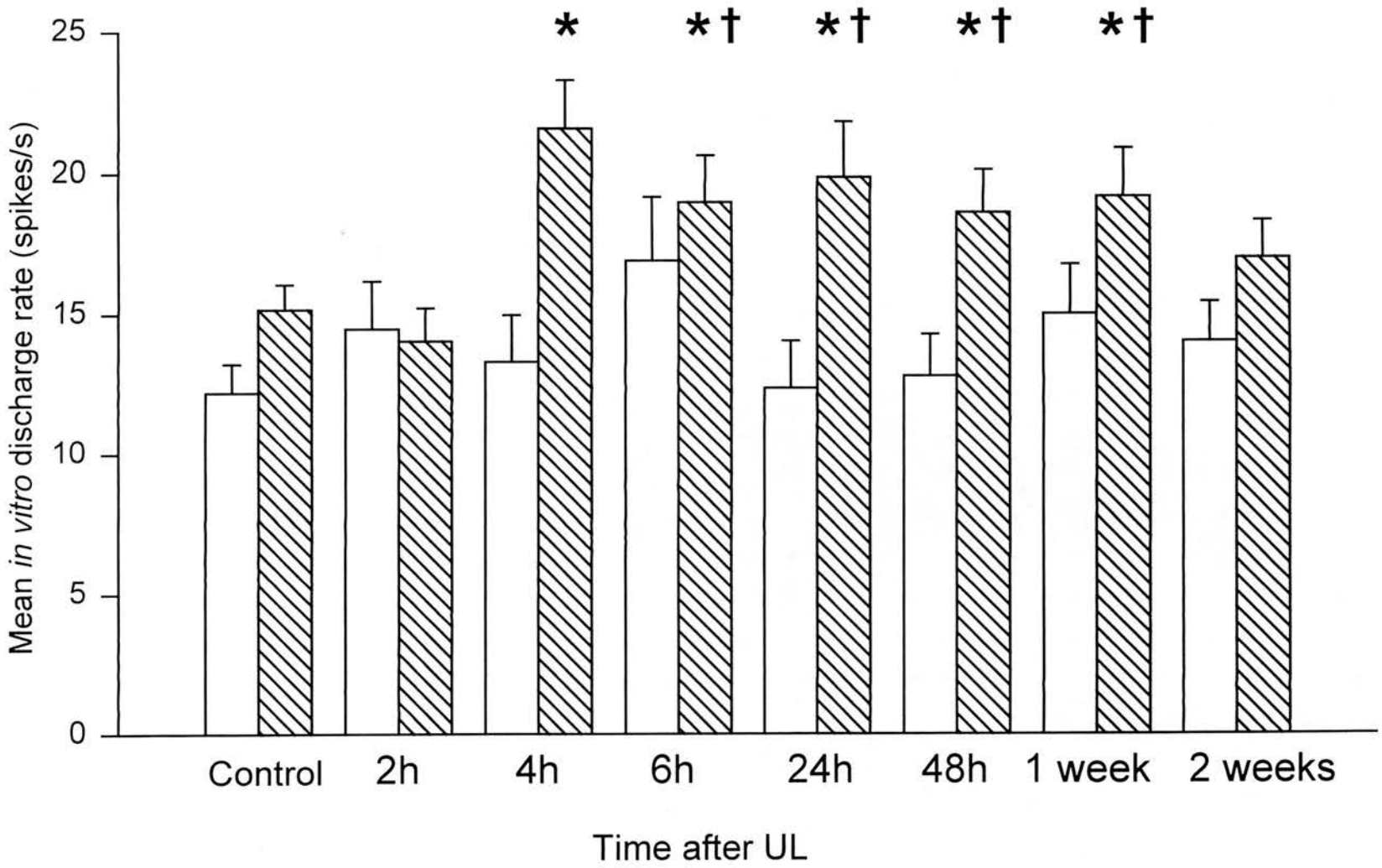

25
20
15
10
5
0
Mean *in vitro* discharge rate (spikes/s)
* *† *† *† *†
Control
2h
4h
6h
24h
48h
1 week
2 weeks
Time after UL

Figure 3.1 **The *in vitro* resting discharge rate of MVN neurones recorded from the ipsilateral nucleus at different times following UL.**

Histograms showing the mean *in vitro* tonic discharge rates ± s.e.m of MVN neurones in the lesioned nucleus in slices prepared at the time stated following unilateral labyrinthectomy. Animals received a left UL under Avertin anaesthesia. Ipsilateral to lesion (left MVN).

Hatched bars represent data from cells recorded in the rostral third of the ipsilateral nucleus.
Open bars represent data from MVN cells recorded from the caudal third of the ipsilateral nucleus.

Statistics: *$p<0.05$ vs. sham rostral cells (MWRST)

†$p<0.05$ vs. contralateral cells of corresponding time point (MWRST)

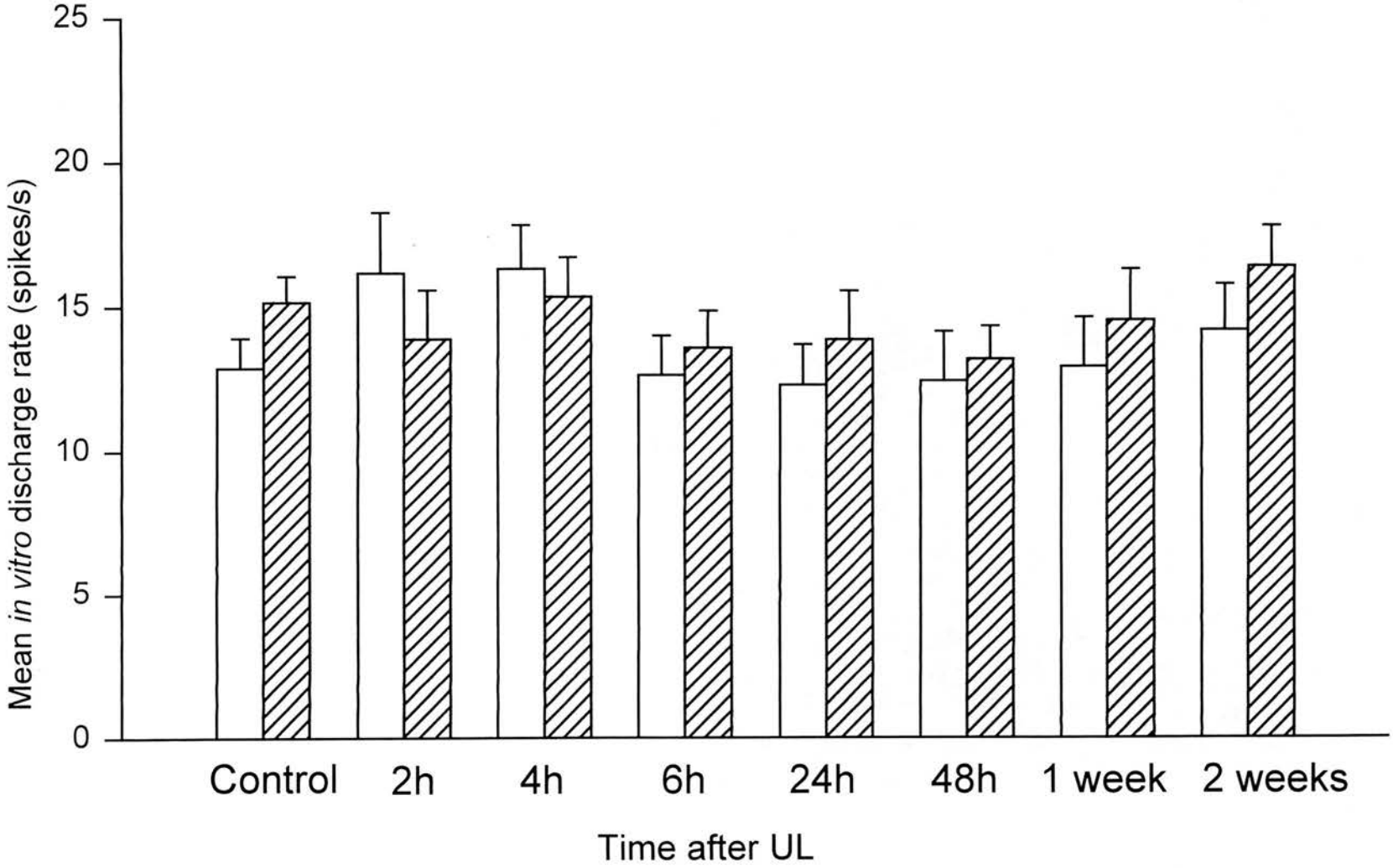

25
20
15
10
5
0
Mean *in vitro* discharge rate (spikes/s)
Control
2h
4h
6h
24h
48h
1 week
2 weeks
Time after UL

Figure 3.2 **The *in vitro* discharge rate of MVN neurones recorded from the contralateral nucleus at different times following UL.**

Histograms showing the mean *in vitro* tonic discharge rates ± s.e.m of MVN neurones in the contralateral nucleus in slices prepared at the time stated following unilateral labyrinthectomy. Animals received a left UL under Avertin anaesthesia.

Hatched bars represent data from MVN cells recorded in the rostral third of the contralateral nucleus.
Open bars represent data from MVN cells recorded from the caudal third of the contralateral nucleus.

No significant differences were found in any region examined.

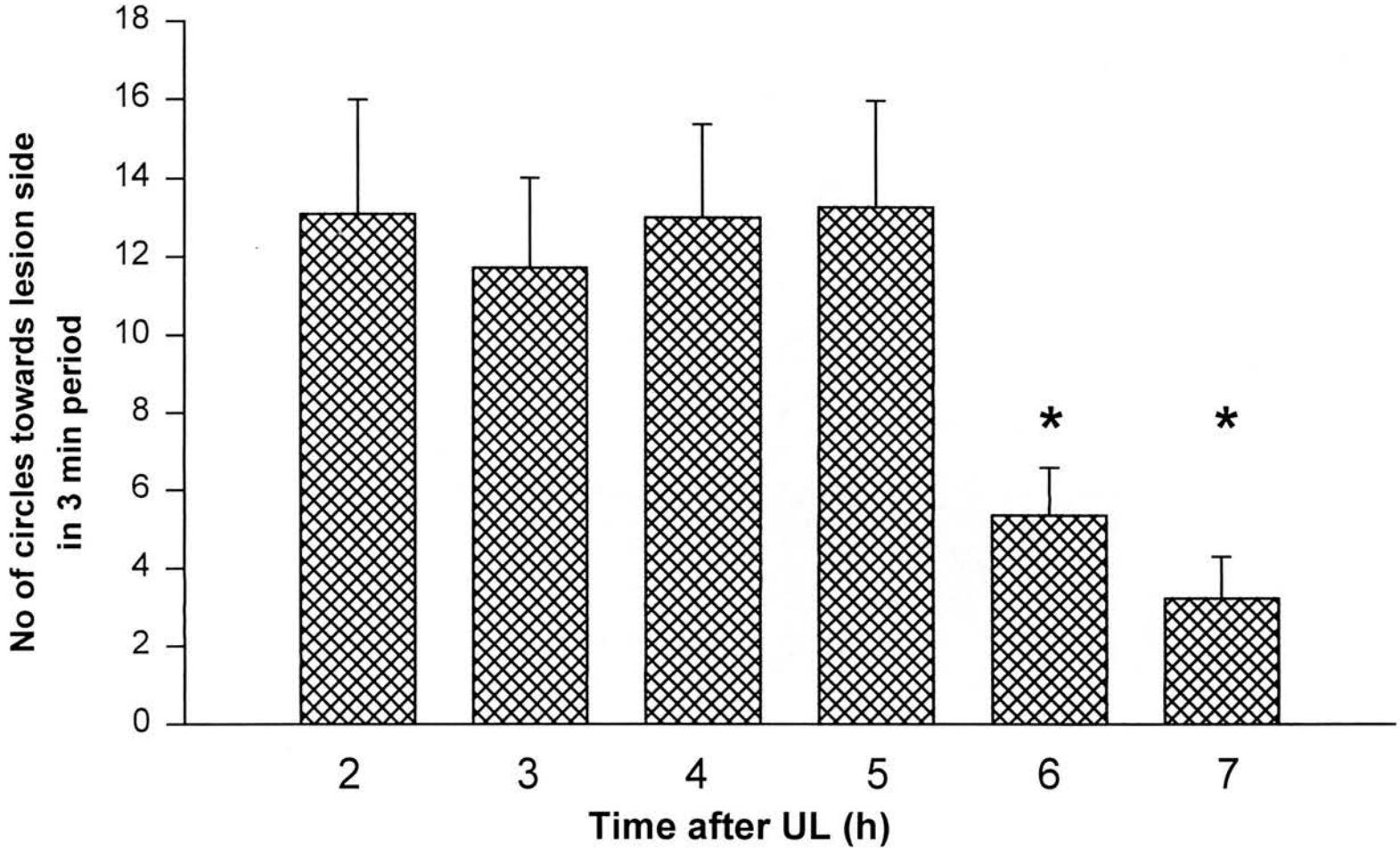

No of circles towards lesion side in 3 min period
18
16
14
12
10
8
6
4
2
0
*
*
2
3
4
5
6
7
Time after UL (h)

Figure 3.3 Quantification of circular walking in rats after UL

Animals received a left UL under Avertin anaesthesia. Circular walking was elicited by placing the animals in an open enclosure, and the number of circles in 3x 3 min periods following a 1 min control period was calculated. Data shown is the mean ± s.e.m for each group of animals at the appropriate time point.

Statistics: *$p<0.001$ one-way RM ANOVA (Neuman Keuls post hoc test)

No. of cells per track
2.5
2.0
1.5
1.0
0.5
0.0
Control
Sham
2h post-UL
4h post-UL
6h post-UL
24h post-UL
48h post-UL
1 week post-UL
2 weeks post-UL
Treatment

Figure 3.4 Number of cells obtained per electrode track in the ipsilateral and contralateral MVN following UL

The number of cells per electrode track was calculated by dividing the total number of cells obtained from an animal group by the total number of electrode tracks made.

Filled circles represents data obtained from the right (labyrinth intact) nucleus.
Filled squares represents data obtained from the left (lesioned labyrinth) nucleus.

No significant differences were observed between cells recorded in the ipsilateral or contralateral MVN.

Coefficent of variance
0.09
0.08
0.07
0.06
0.05
0.04
Control
2h post-UL
4h post-UL
6h post-UL
24h post-UL
48h post-UL
1 week post-UL
2 weeks post-UL
Treatment

Figure 3.5 **Coefficient of variance values for rostral MVN neurones recorded from animals at varying time points following UL**

Mean ± s.e.m. coefficients of variance obtained from rostral cells from animals at the time stated following UL.

Filled circles represent data from rostral cells recorded from the right (labyrinth intact) nucleus.
Filled squares represent data from rostral cells recorded from the left (lesioned labyrinth) nucleus.

No significant differences were found between rostral MVN neurones recorded from either the ipsilateral or contralateral nuclei at any time point examined.

Coefficent of variance
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
Control
2h post-UL
4h post-UL
6h post-UL
24h post-UL
48h post-UL
1 week post-UL
2 weeks post-UL
Treatment

Figure 3.6 **Coefficient of variance values for caudal MVN neurones recorded from animals at varying time points following UL**

Mean ± s.e.m. coefficients of variance obtained from caudal cells from animals at varying time points following UL.

Filled circles represent data from rostral cells recorded from the right (labyrinth intact) nucleus.
Filled squares represent data from rostral cells recorded from the left (lesioned labyrinth) nucleus.

No significant differences were found between caudal MVN neurones recorded from either the ipsilateral or contralateral nuclei at any time point examined.

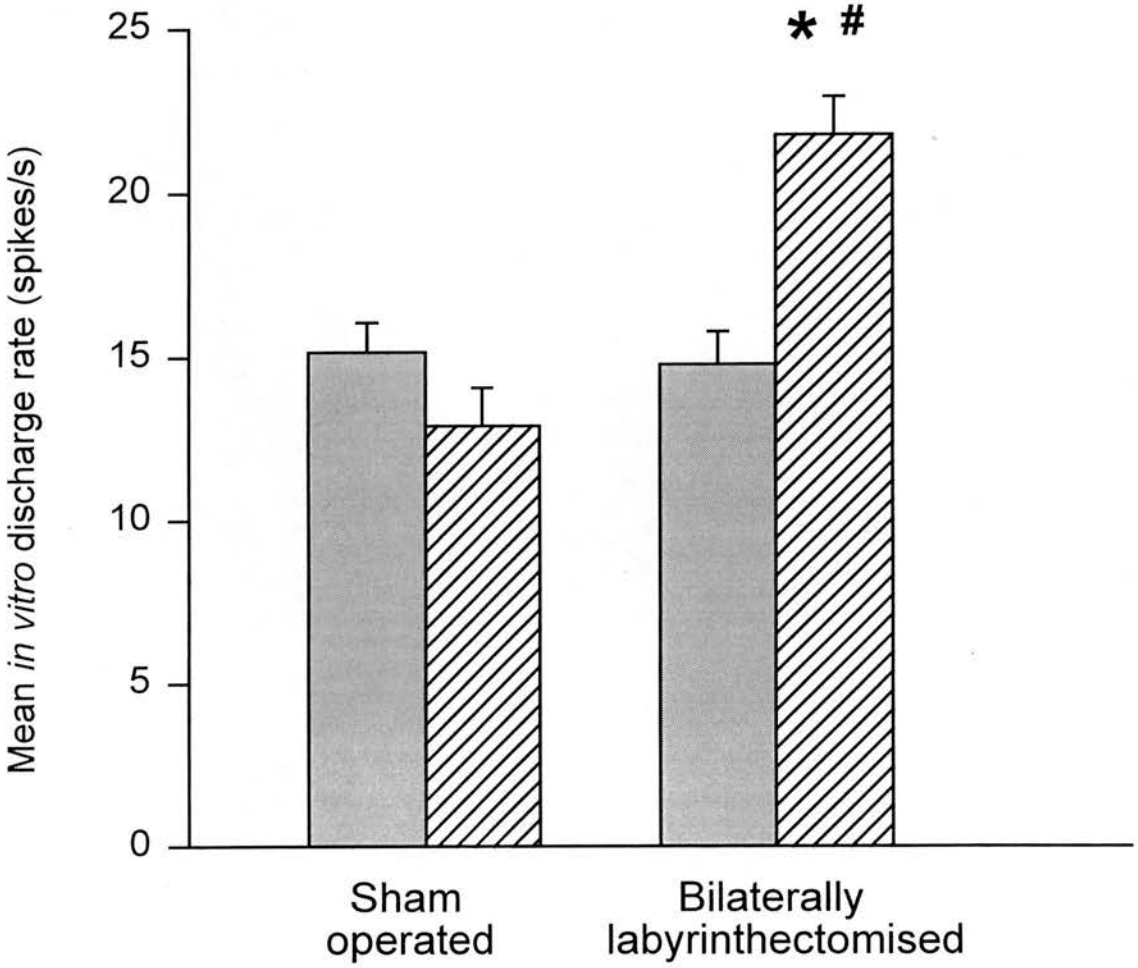

25
20
15
10
5
0
Mean *in vitro* discharge rate (spikes/s)
* #
Sham operated
Bilaterally labyrinthectomised

Figure 3.7 **The *in vitro* discharge rate of MVN neurones recorded from sham-operated animals and animals 4h following bilateral labyrinthectomy**

Histogram showing the mean *in vitro* tonic discharge rates (± s.e.m) of MVN neurones in nuclei in slices prepared from bilaterally-labyrinthectomised animals 4h following surgery.

Filled bars represent data from cells recorded in the rostral third of the MVN
Hatched bars represent data from cells recorded in the caudal third of the MVN

Statistics: *$p<0.0001$ compared to control caudal cells (MWRST)
#$p<0.0001$ compared to rostral cells in the corresponding nuclei (MWRST)

PART 2 Changes in the responsiveness of MVN cells to muscimol and baclofen after UL

It has been suggested that GABA may play a role in the process of VC. A decrease in benzodiazepine binding in the ipsilateral MVN within 3h post-UL was demonstrated by Calza *et al.*, (1992), and an increase in the level of GABA detected in the VNC 3-6 days following UL was demonstrated by Thompson *et al.* (1986). The responsiveness of MVN neurones to GABA was investigated by bath application of the $GABA_A$ agonist muscimol, and the $GABA_B$ agonist baclofen to slices containing the MVN prepared from animals 4h post-UL. The experiments described in this part of the thesis were performed with the help of Dr. Toshiaki Yamanaka who visited the laboratory in May 1997 for 1 year.

Increased excitability of rostral cells in the lesioned MVN following UL

A total of 278 MVN cells were recorded in this series of experiments. The mean discharge rate of MVN cells recorded in the rostral region of the nucleus ipsilateral to the lesion of animals which had received a left UL 4h previously was significantly higher than rostral MVN cells recorded in the corresponding region of MVN slices prepared from control animals in this series of experiments (21.46 ± 1.11 spikes/s, n=63 cells versus 16.19 ± 1.26 spikes/s, n=40 cells). The mean discharge rate of MVN cells in the caudal region of the lesioned nucleus, and the rostral and caudal parts of the contralateral nucleus, were not different from those in the corresponding regions of slices from control animals (ipsilateral caudal cells, 17.33 ± 1.03 spikes/s, n=45 cells versus 17.04 ± 1.03 spikes/s, n=37 cells in controls; contralateral rostral cells, 16.85 ± 1.09 spikes/s, n=50 cells, and contralateral caudal cells, 16.73 ± 1.04 spikes/s, n=43 cells). These results confirm the findings in Part 1 of this chapter, that there is a significant increase in the excitability of MVN neurones in the rostral third of the lesioned nucleus at 4h post-UL.

Responsiveness of MVN cells to muscimol and baclofen *in vitro*

In slices prepared from normal animals, bath application of the $GABA_A$ agonist muscimol (1-100μM for 60 seconds, n=28cells) and the $GABA_B$ agonist baclofen (1-30μM for 60 seconds, n=38cells) caused a reversible, dose-dependent inhibition of the resting discharge rate of all MVN cells tested (figure 3.8). Table 3.2 shows the parameters of the logistic curves fitted to the dose-response relationship (maximal response, mid-point concentration (EC50), and slope) for all the data in these experiments. The effects of muscimol and baclofen on both rostral and caudal MVN cells in slices from control animals did not differ as illustrated by their respective dose response curves in fig 3.9, 3.10, 3.11, and 3.12.

In contrast, the response of MVN cells to $GABA_A$ and $GABA_B$ agonists in both the rostral and caudal thirds of nuclei taken from animals which had received a labyrinthectomy 4 hours previously were different from the controls.

Ipsilateral MVN

Rostral cells in the lesioned MVN at 4h post-UL showed a marked decrease in their responsiveness to muscimol at all concentrations tested. This is seen as a significant rightward shift of the dose-response relationship for rostral cells compared to control cells (Figure 3.9 $p<0.05$, 2 way ANOVA). The EC50 value (K) of the fitted dose-response curve increased from 8.95 ± 0.21μM, n=14 cells in MVN slices from control animals to 45.3 ± 9.46μM, n=10 cells after UL. This was accompanied by a significant decrease in the logistic slope factor (P=0.776 ± 0.0062) in slices from UL animals compared with 1.138 ± 0.016 in slices from control animals ($p<0.005$, 2 way ANOVA).

In the caudal region of the lesioned MVN, there was no significant shift in the EC50 value for the fitted dose-response relationship (K=10.54 ± 3.45 μM, n=14 cells after UL compared with 13.0 ± 3.52 μM, n=12 cells from control animals) nor in the slope factor (P= 0.793 ± 0.123 after UL compared with 0.944 ± 0.09). However at higher doses of muscimol (30 and 100 μM) the mean inhibition of the tonic

discharge rate was significantly reduced compared to normal ($p<0.05$, two-tailed Student's t-test). This is seen as a significant rightward shift of the dose-response relationship for rostral cells compared to control cells.

The decreased responsiveness of rostral MVN cells in the lesioned nucleus to muscimol was accompanied by changes in the time-course of the inhibitory response. As illustrated in figure 3.8, the inhibitory effect of muscimol was slower to develop and the inhibition of the firing rate following the 60sec period of drug application was longer in duration in cells from the lesioned nucleus compared to MVN cells from control animals. This effect was quantified by measuring the mean time taken to reach maximal inhibition following the 60 sec pulse of muscimol. As can be seen in figure 3.13 in lesioned rostral MVN cells the time taken to reach maximal inhibition was significantly longer than that of control cells at doses of 10μM and 30μM muscimol (control cells 66.9 ± 7.06 sec at 10μM, n= 14 cells; 122 ± 15.7 sec at 30μM, n=10 cells; compared with 144 ± 43.6 sec at 10μM, n=10 cells; and 299 ± 67.4 sec at 30μM, n=9 cells in ipsilateral rostral MVN cells ($P<0.05$ 2-way ANOVA),

These changes in the functional efficacy of GABA receptors in rostral ipsilateral MVN cells are thus accompanied by a significant increase in the resting discharge in this area, however, the changes in GABA receptor efficacy observed in the caudal region, are not accompanied by changes in the resting discharge rate.

Contralateral MVN

In the contralateral MVN, the dose-response relationship for muscimol in the rostral region of the nucleus showed no significant difference in either the EC50 value or the slope factor after UL compared to cells from control animals (Figure 3.11). However, in the caudal region of the contralateral nucleus, there was a significant increased responsiveness of MVN cells to muscimol, which was seen as a significant leftward shift of the dose-response relationship ($p<0.05$, two-way ANOVA, Fig 3.12). The EC50 of the fitted dose-response curve decreased

significantly from 13.0 ± 3.52, n=14 cells in controls to 2.48 ± 0.51, n=22 cells after UL ($p<0.05$), and this was accompanied by a significant increase in slope (E=1.342 ± 0.298 compared with 0.944 ± 0.09 in controls, $p<0.05$). The changes in GABA receptor efficacy in the caudal MVN cells of the contralateral nucleus were not accompanied by any changes in resting discharge rate.

In contralateral rostral MVN cells the time taken to reach maximal inhibition was not different from control cells (control cells 53.3 ± 6.41 sec at 3μM n=10 cells; 66.9 ± 7.06 sec at 10μM, n=14 cells and 122 ± 15.7 sec at 30μM,n=10 cells compared with 50.4 ± 2.25 sec at 3μM n=22 cells; 65.9 ± 5.03 sec at 10μM n=16 cells and 118 ± 22.8 sec at 30μM n=5 cells in contralateral rostral MVN cells Figure 3.13 Table 3.3a).

Responsiveness of ipsilateral and contralateral MVN cells to baclofen 4h after UL

Ipsilateral MVN

Cells in both the rostral and caudal regions of the lesioned MVN, showed a decreased responsiveness to the $GABA_B$ agonist baclofen compared to control values. The dose-response relationship for rostral MVN cells showed a significant rightward shift compared to controls ($p<0.05$, two-way ANOVA). The EC50 value increased from 5.02 ± 0.32μM, n=16 cells in controls to 22.7 ± 6.2 μM, n=27 cells after UL ($p<0.05$), this was accompanied by a significant decrease in the slope of the fitted logistic curve (E=0.575 ± 0.049 after UL compared with 0.798 ± 0.023 in controls $p<0.05$). In the caudal MVN cells of the lesioned nucleus, the parameters of the fitted dose-response curves for baclofen did not differ significantly from control (table 3.2).

Contralateral MVN

In the contralateral MVN, there was a small but significant increased responsiveness of both rostral and caudal cells to baclofen. In the rostral MVN, the EC50 value for the dose-response curve decreased from 5.02 ± 0.32, n=16 cells in

controls to 2.18 ± 0.33 μM, n= 20 cells after UL ($p<0.05$), without a significant change in slope. In the caudal region of the MVN the EC50 value decreased from 2.92 ± 0.45, n=18 cells to 1.54 ± 0.08 μM, n=20 cells after UL ($p<0.05$), again without a significant change in slope. There were no changes in the time taken to reach maximal inhibition in response to baclofen in any of the cells looked at (Figure 3.14, table 3.3b).

	Muscimol			**Baclofen**		
	MAX	EC50(μM)	SLOPE	MAX	EC50(μM)	SLOPE
1.Rostral	107.9 ± 1.1	8.95 ± 0.21	1.13 ± 0.01	114.7 ± 2.3	5.02 ± 0.32	0.79 ± 0.02
1. Caudal	132.5 ± 14.6	13.0 ± 3.5	0.94 ±0.09	103.4 ± 5.3	2.92 ± 0.45	0.86 ± 0.09
2. Rostral	123.4 ± 7.7	**45.3 ± 9.46**	**0.78 ± 0.01**	114.2 ± 7.1	**22.7 ± 6.2**	**0.58 ± 0.05**
2. Caudal	105.3 ± 10.2	10.5 ± 3.45	0.79±0.123	97.3 ± 12.6	3.85 ± 1.89	0.69 ± 0.2
3. Rostral	112.9 ± 14.8	8.07 ± 2.9	0.93 ± 0.14	**94.5 ± 4.6**	**2.18 ± 0.33**	1.02 ± 0.12
3. Caudal	101.4 ± 7.5	**2.48 ± 0.51**	1.34 ± 0.29	95.1 ± 1.5	**1.54 ± 0.08**	0.92 ± 0.04

Table 3.2

Summary of maximum inhibition obtained from fitted curve to dose-response curve, EC50 and slope of:

1) MVN cells recorded from slices prepared from control animals.

2) neurones recorded from ipsilateral MVN slices prepared from animals which had received a labyrinthectomy 4h previously.

3) contralateral labyrinthectomised MVN neurones.

Values in bold indicate significant differences when compared to corresponding control values ($p<0.05$ Students t-test).

DOSE	CONTROL MVN	IPSILATERAL UL. MVN	CONTRALATERAL UL. MVN
ROSTRAL CELLS			
3μM	53.3 ± 6.41	68.7 ± 10.2	50.4 ± 2.25
10μM	66.9 ± 7.06	**144 ± 43.6**	65.9 ± 5.03
30μM	122 ± 15.7	**299 ± 67.4**	118 ± 22.8
CAUDAL CELLS			
3μM	49.7 ± 4.48	62 ± 6.41	47.2 ± 2.61
10μM	65.8 ± 8.92	91.1 ± 19.9	53.1 ± 5.12
30μM	136 ± 22.9	227 ± 62	76.3 ± 5.9

Table 3.3a

Mean time taken to reach maximum inhibition (sec ± s.e.m.) in response to single 60sec pulse of muscimol. Values in bold indicate significantly different when compared to corresponding control values ($p<0.05$ Students t-test).

DOSE	CONTROL MVN	IPSILATERAL UL. MVN	CONTRALATERAL UL. MVN
ROSTRAL CELLS			
1μM	66.07 ± 5.9	59.82 ± 5.2	68.89 ± 3.16
3μM	74.2 ± 5.8	76.7 ± 4.59	72.2 ± 3.4
10μM	92.9 ± 8.9	94.1 ± 7.03	85.1 ± 3.17
CAUDAL CELLS			
1μM	49.13 ± 3.7	55 ± 3.95	63.4 ± 2.80
3μM	74.7 ± 5.61	69.5 ± 4.25	78.9 ± 5.25
10μM	88.1 ± 7.29	89.3 ± 5.5	86.5 ± 6.54

Table 3.3b

Mean time taken to reach maximum inhibition (sec ± s.e.m.) in response to single 60sec pulse of baclofen.

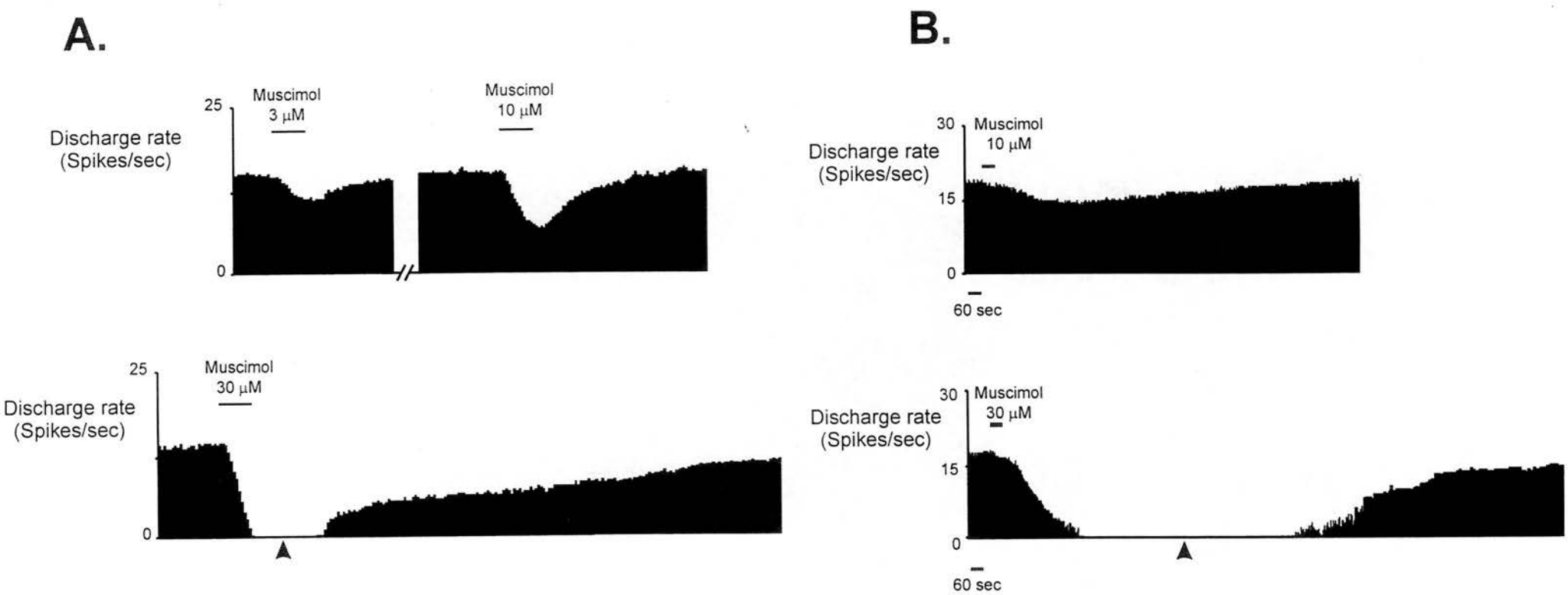

A.
Discharge rate
(Spikes/sec)
25
0
Muscimol
3 μM
Muscimol
10 μM
Discharge rate
(Spikes/sec)
25
0
Muscimol
30 μM
B.
Discharge rate
(Spikes/sec)
30
15
0
Muscimol
10 μM
60 sec
Discharge rate
(Spikes/sec)
30
15
0
Muscimol
30 μM
60 sec

Figure 3.8 Response of tonically active MVN cells to the $GABA_A$ agonist, muscimol.

A. Response of control MVN cell located in the rostral third of the nucleus to 3, 10 and 30 μM muscimol.

B. Response of MVN cell recorded from the rostral third of the lesioned nucleus taken from an animal which had received a UL 4h previously. Note the inhibitory effect of muscimol is slower to develop and the inhibition of firing is longer in duration compared to cell in panel A.

In both A and B the 60s drug application is indicated by the bar above the data.

When a cell goes silent following drug application, the time taken to reach maximum inhibition is assumed to be the midpoint of the silencing. This is indicated by the arrow in the lower panels of A and B

Ipsilateral rostral MVN cells

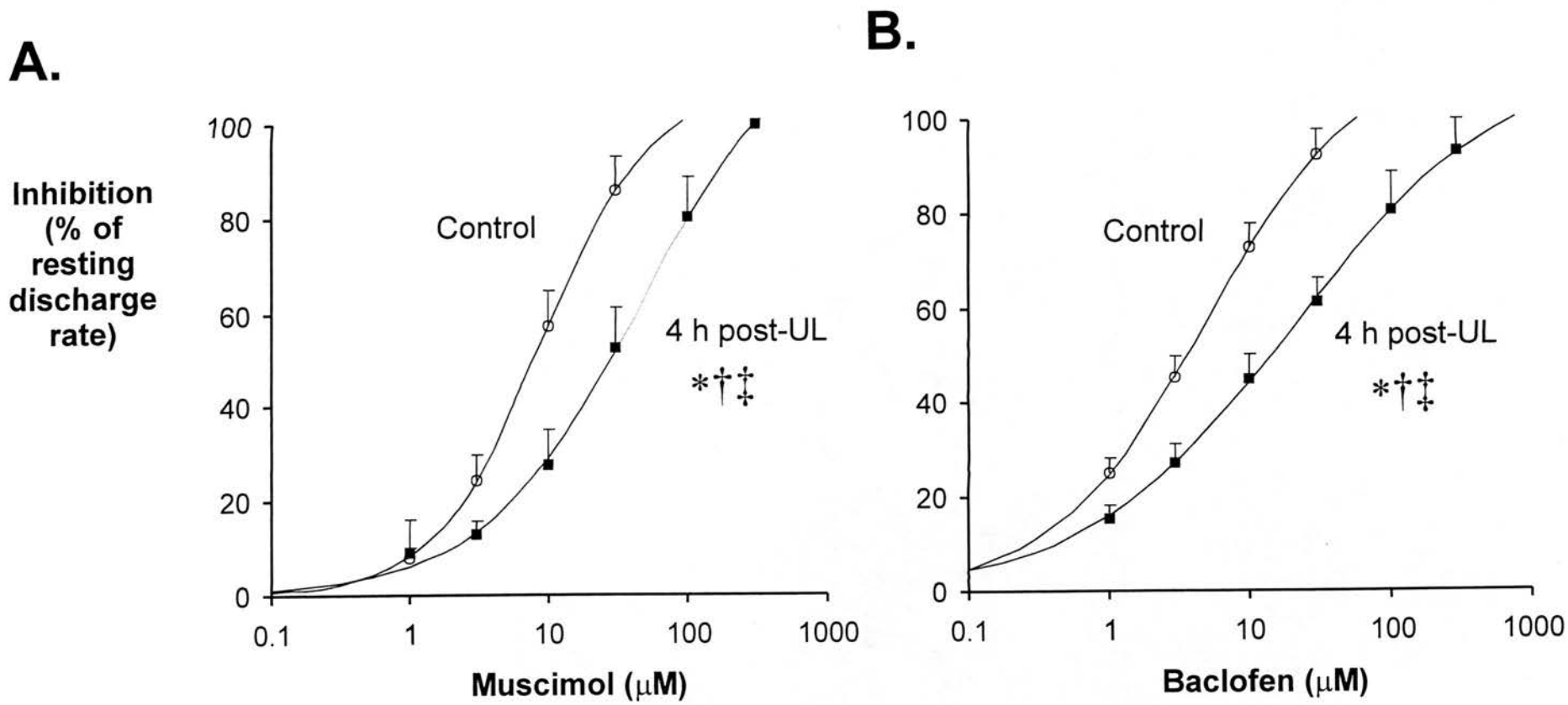


Figure 3.9 Fitted dose-response curves of ipsilateral rostral MVN neurones 4h post-UL to muscimol and baclofen

Animals received a left UL under Avertin anaesthesia. 4h post-UL slices containing the ipsilateral MVN were explored for changes in responsiveness to GABA agonists. Each data point represents the mean percentage inhibition for each concentration and dose response curves of y=Max*(p/(p+EC50)), were fitted to this data.

A. Fitted dose response curve for $GABA_A$ agonist muscimol

B. Fitted dose response curve for $GABA_B$ agonist baclofen

Open circles represent data from control rostral MVN cells

Filled squares represent data from ipsilateral rostral MVN cells recorded 4h post-UL

Statistics: *$p<0.05$, vs. dose response curve for control rostral MVN cells. 2-way ANOVA

†$p<0.005$, vs. EC50 for control rostral cells. 2-way ANOVA

‡$p<0.005$, vs. slope of control rostral cells. 2-way ANOVA

Ipsilateral caudal MVN cells

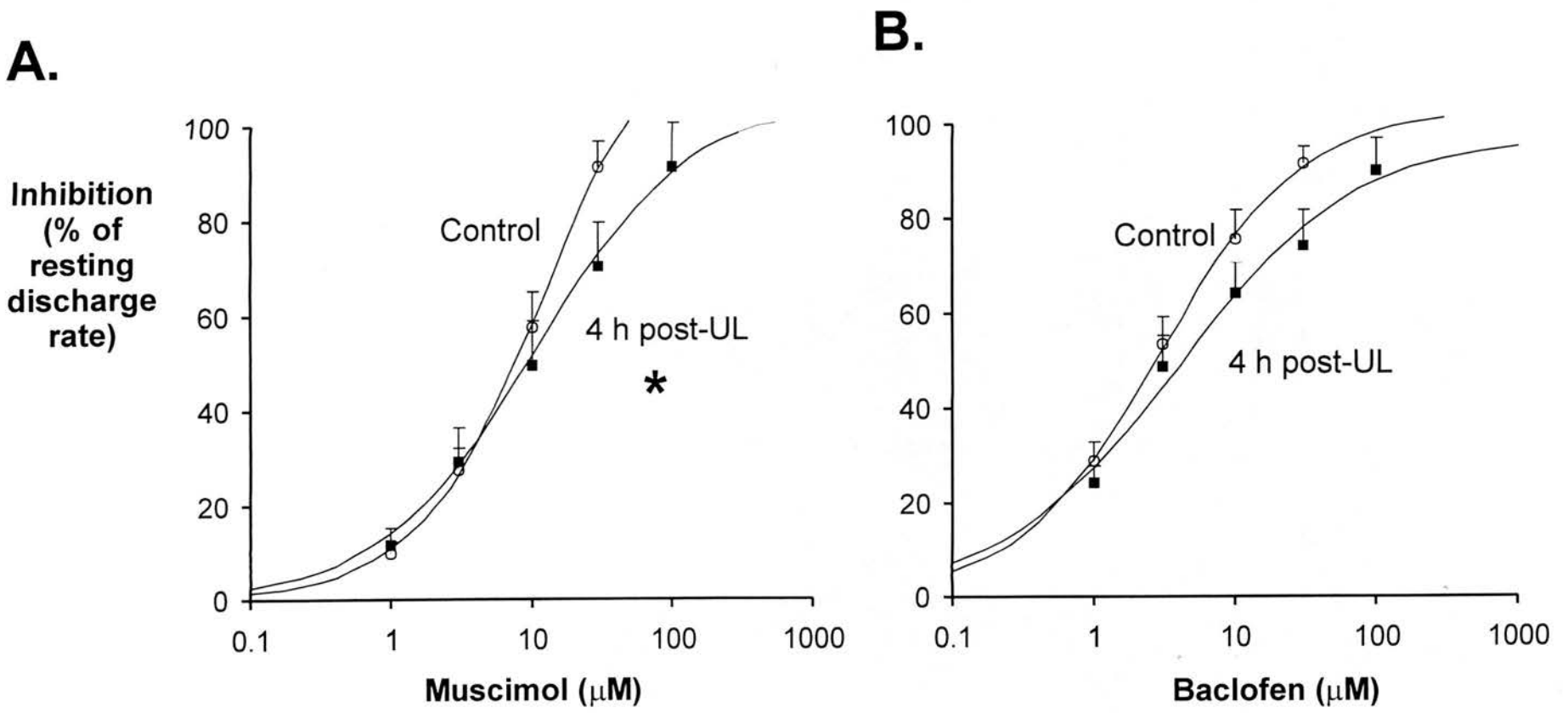


Figure 3.10 Fitted dose-response curves of ipsilateral caudal MVN neurones 4h post-UL to muscimol and baclofen

Animals received a left UL under Avertin anaesthesia. 4h post-UL slices containing the ipsilateral MVN were explored for changes in responsiveness to GABA agonists. Each data point represents the mean percentage inhibition for each concentration and fitted dose response curves of y=Max*(p/(p+EC50)), were fitted to this data

A. Dose response curve for $GABA_A$ agonist muscimol

B. Dose response curve for $GABA_B$ agonist baclofen

Open circles represent data from control caudal MVN cells

Filled squares represent data from ipsilateral caudal MVN cells recorded 4h post-UL

Statistics: $^*p<0.05$, vs. dose response curve for control caudal MVN cells at higher doses. 2-way ANOVA

Contralateral rostral MVN

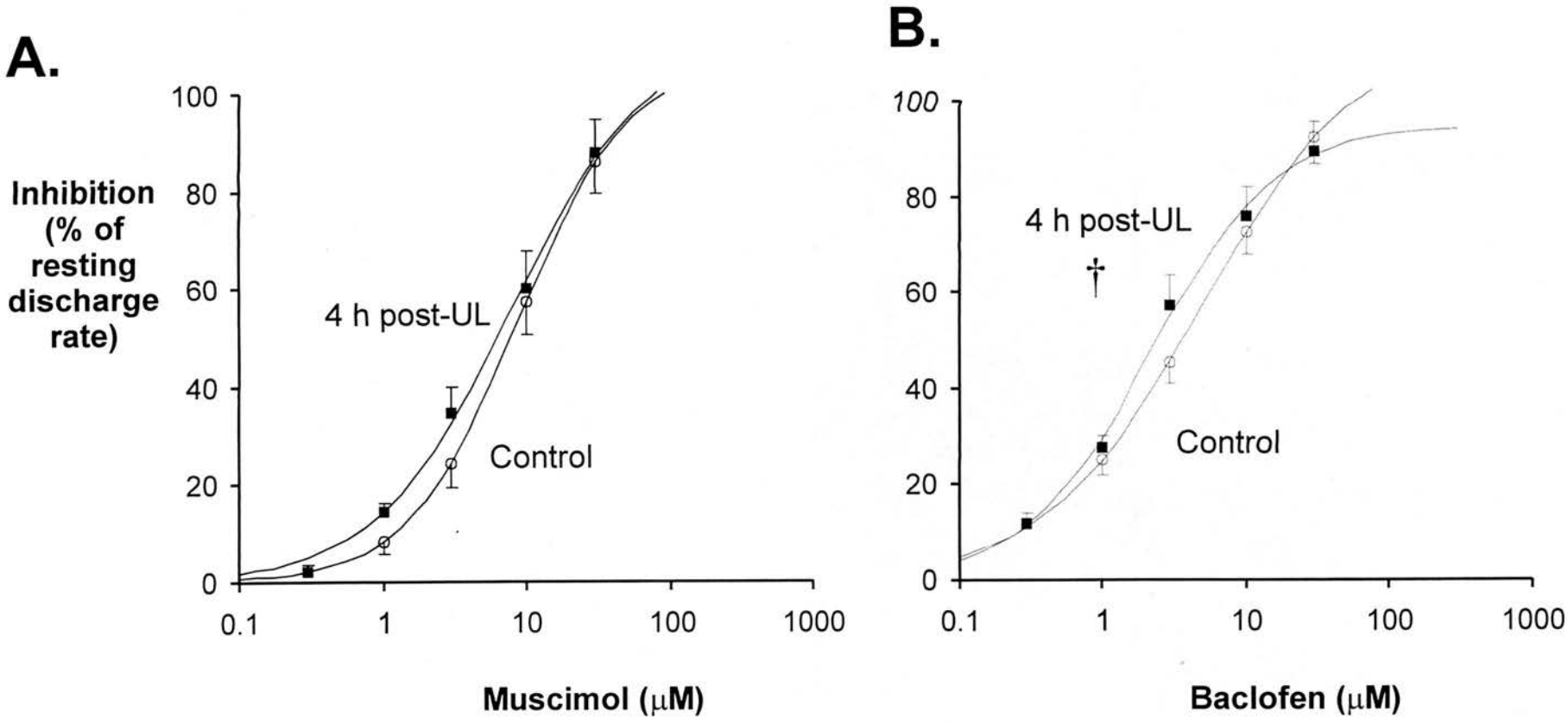


Figure 3.11 Fitted dose-response curves of contralateral rostral MVN neurones 4h post-UL to muscimol and baclofen

Animals received a left UL under Avertin anaesthesia. 4h post-UL slices containing the contralateral MVN were explored for changes in responsiveness to GABA agonists. Each data point represents the mean percentage inhibition for each concentration and fitted dose response curves of y=Max*(p/(p+EC50)), were fitted to this data

A. Dose response curve for $GABA_A$ agonist muscimol

B. Dose response curve for $GABA_B$ agonist baclofen

Open circles represent data from control rostral MVN cells

Filled squares represent data from contralateral rostral MVN cells recorded 4h post-UL

Statistics: †$p<0.05$, vs. EC50 for control rostral cells. 2-way ANOVA

Contralateral caudal MVN

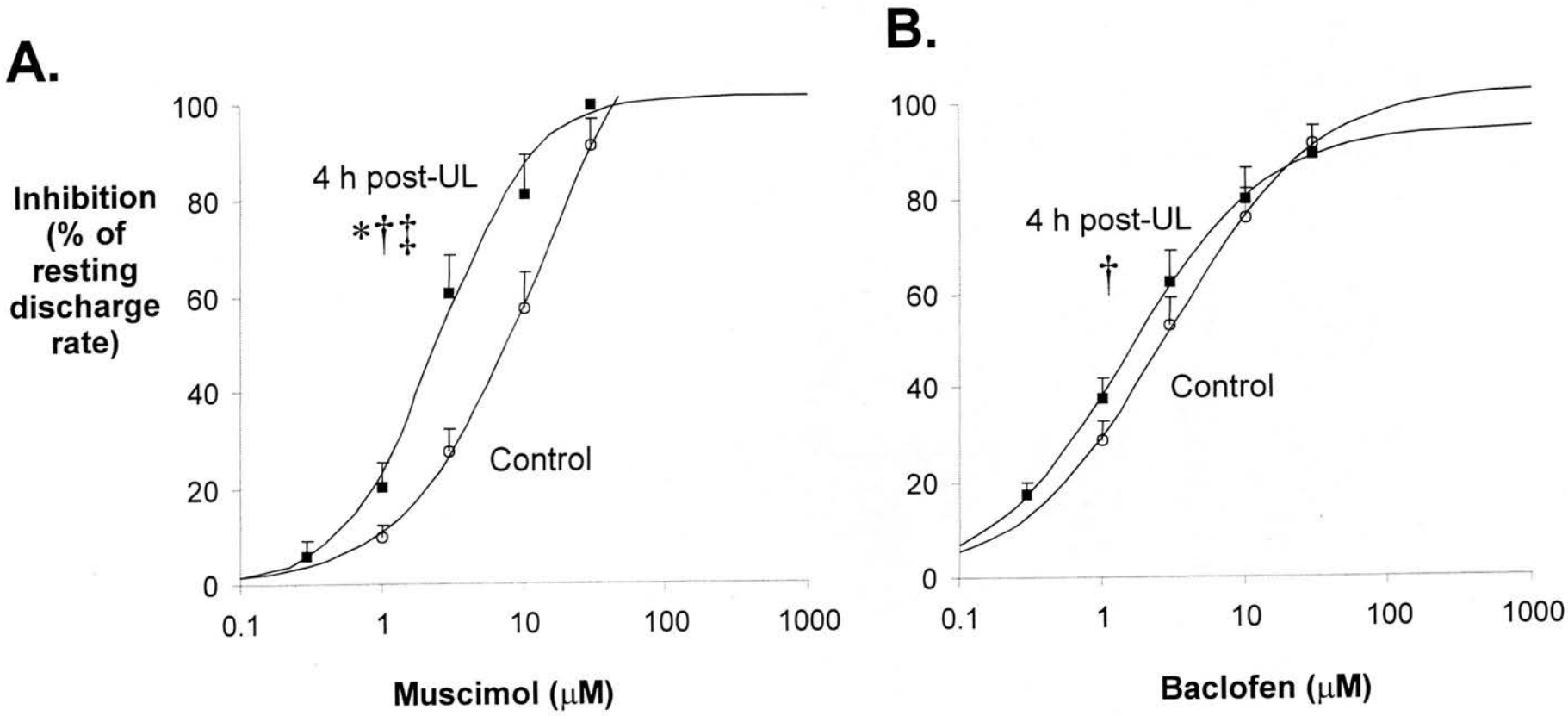


Figure 3.12 Fitted dose-response curves of contralateral caudal MVN neurones 4h post-UL to muscimol and baclofen

Animals received a left UL under Avertin anaesthesia. 4h post-UL slices containing the contralateral MVN were explored for changes in responsiveness to GABA agonists. Each data point represents the mean percentage inhibition for each concentration and fitted dose response curves of y=Max*(p/(p+EC50)), were fitted to this data

A. Dose response curve for $GABA_A$ agonist muscimol

B. Dose response curve for $GABA_B$ agonist baclofen

Open circles represent data from control caudal MVN cells

Filled squares represent data from contralateral caudal MVN cells recorded 4h post-UL

Statistics: *p<0.05, vs. dose response curve for control rostral MVN cells. 2-way ANOVA

†p<0.05, vs. EC50 for control caudal cells. 2-way ANOVA

‡p<0.05, vs. slope of control caudal cells. 2-way ANOVA

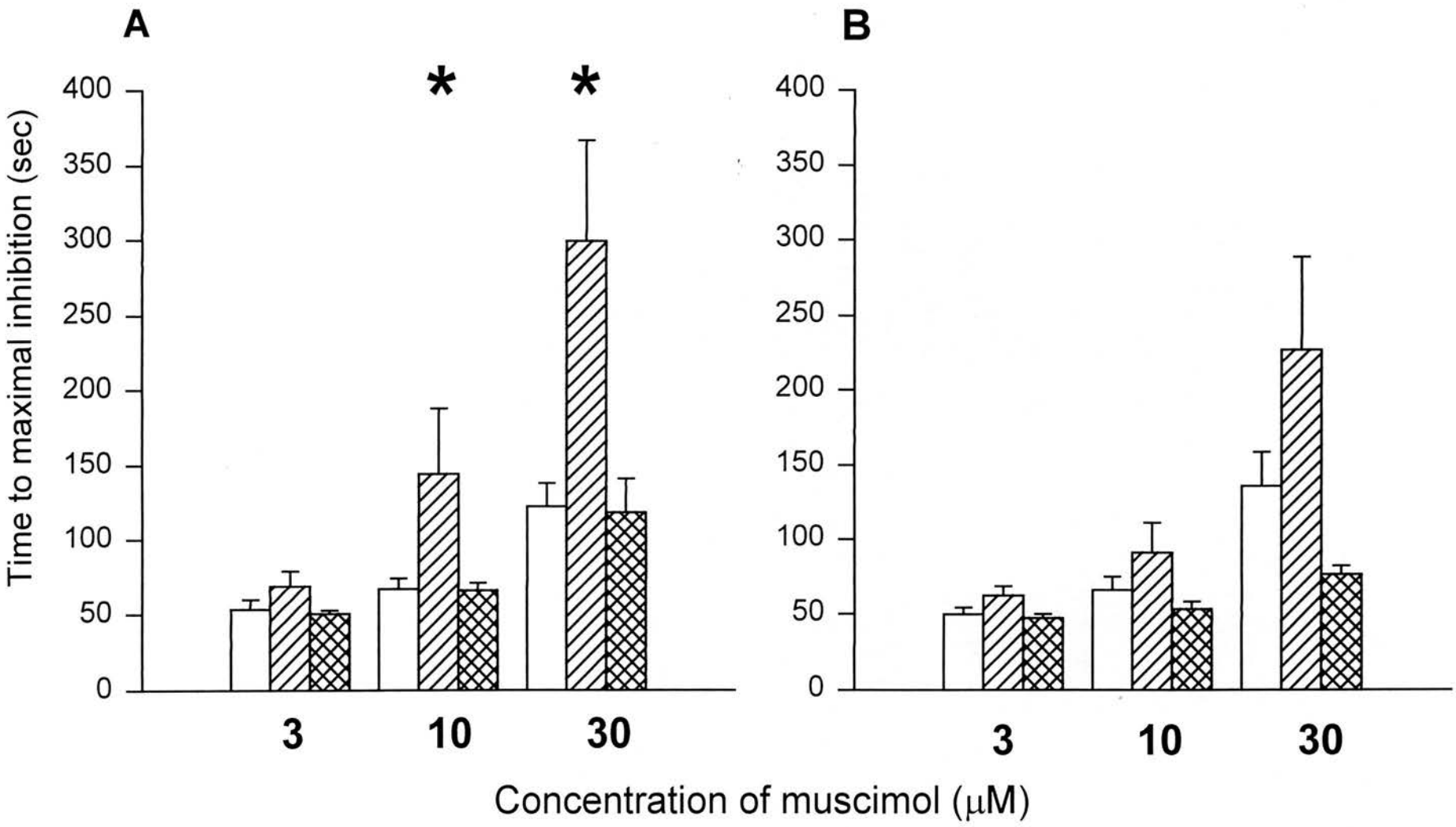

A
B
*
*
400
350
300
250
200
150
100
50
0
Time to maximal inhibition (sec)
3
10
30
Concentration of muscimol (μM)

Figure 3.13 **Time to maximum inhibition for control, contralateral, and ipsilateral rostral MVN cells 4h following UL**

Histograms showing the time taken to reach maximum inhibition in response to the stated doses of the $GABA_A$ agonist muscimol.

A. Time to maximum inhibition for rostral MVN cells

B. Time to maximum inhibition for caudal MVN cells

Open bars represent data from MVN cells recorded from control animals

Single hatched bars represent data from MVN cells recorded from the contralateral nucleus of animals 4h post-UL

Double hatched bars represent data from MVN cells recorded from the ipsilateral nucleus of animals 4h post-UL

Statistics: $^{*}p<0.05$, 2-way ANOVA

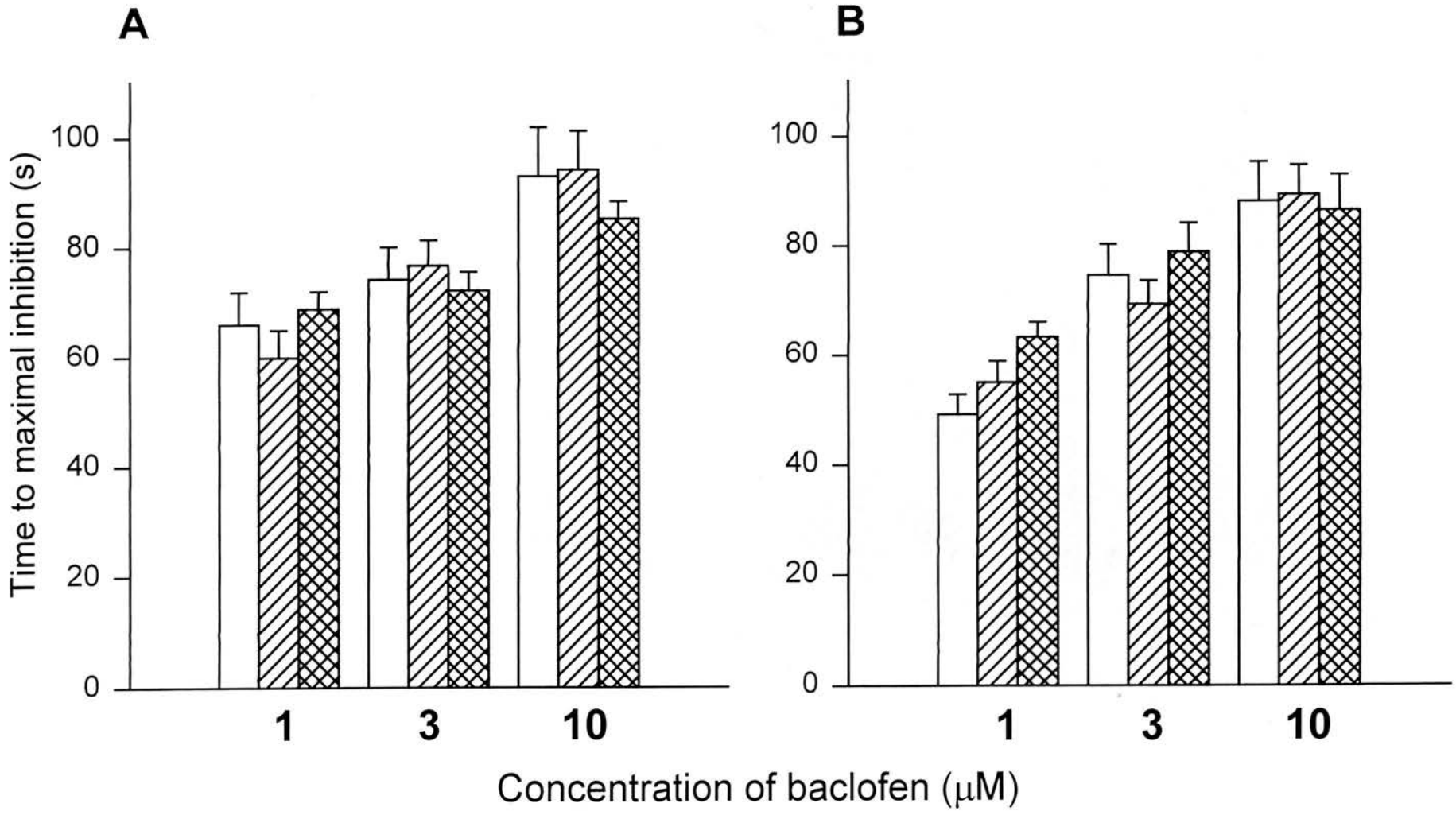

A
B
Time to maximal inhibition (s)
100
80
60
40
20
0
1
3
10
Concentration of baclofen (μM)

Figure 3.14 **Time to maximum inhibition for control, contralateral, and ipsilateral rostral MVN cells**

Histograms showing the time taken to reach maximum inhibition in response to the stated doses of the $GABA_B$ agonist baclofen

A. Time to maximum inhibition for rostral MVN cells

B. Time to maximum inhibition for caudal MVN cells

Open bars represent data from MVN cells recorded from control animals

Single hatched bars represent data from MVN cells recorded from the contralateral nucleus of animals 4h post-UL

Double hatched bars represent data from MVN cells recorded from the ipsilateral nucleus of animals 4h post-UL

No statistical differences were found between any groups tested.

3.4 DISCUSSION

Changes in excitability of MVN neurones in vitro following UL

The results of the first set of experiments demonstrated that there was a significant increase in the mean resting discharge rate of MVN neurones in the rostral third of the lesioned nucleus following UL. The increase in discharge rate was first observed at 4 hours post-UL and remained significantly high until 1 week after surgery. This is the first study of its kind to systematically study the changes in firing rates of MVN cells *in vitro* during the period when the static behavioural symptoms are at their most severe. In the only other *in vitro* study, Smith and Darlington (1992), observed that the firing rates of ipsilateral MVN cells were higher than those of a previous study on normal guinea pig cells. However, in this study recordings were made 3 days to 2 months following UL at a time when the static behavioural deficits of VC had compensated.

During the first 12h *in vivo* MVN cells ipsilateral to the lesion have been found to be silent (Smith and Curthoys, 1988a; Ris et al., 1995), and this has been suggested to be due to an increase in the amount of commissural inhibition to which these cells are subject to immediately after UL. The primary vestibular afferents of the vestibular nerve predominantly terminate in the rostral area of the MVN (Stein and Carpenter, 1967; Gacek, 1969; Korte and Friedrich, 1979; Carlton and Carpenter, 1984; Sato et al., 1989). This area also receives a major commissural projection from the rostral area of the contralateral MVN (Newlands et al., 1989). Recently, Darlington and Smith (1996) suggested that the silencing of MVN cells in the lesioned nucleus *in vivo* is a result of 'neural shock' caused by elevated Ca^{2+} levels in MVN cells. If the MVN cells are silenced by neural shock in this way, it would be predicted that there would be no neural activity detected in *in vitro* slices at the early time points following UL. However, the results here show that ipsilateral MVN cells are not silent *in vitro,* even at 2h post-UL when the vestibular symptoms *in vivo* are near their maximal intensity. This then supports the view that the silencing of the

MVN cells *in vivo* is not due to neural shock but to disfacilitation following the loss of their primary afferent input and increased inhibition from the vestibular commissures and/or the inhibitory pathway from the cerebellum (Anguat and Brodal, 1967; Brodal, 1974).

While several possible mechanisms may underlie the increase in excitability of these cells after UL, the available evidence suggests that the increase in excitability of ipsilateral MVN cells after UL may be due to the desensitisation or down-regulation of GABA receptors on them, in response to the excessive commissural or cerebellar inhibition to which they are subjected over several hours in vivo, so that subsequently in vitro they are less responsive to the local release of GABA and show higher resting discharge rates than normal.

Changes in GABA receptor efficacy in the MVN following UL

The results from the second set of experiments in this study demonstrated, that in the lesioned MVN there is a decrease in responsiveness to GABA suggesting that there are rapid compensatory changes in the functional efficacy of $GABA_A$ and $GABA_B$ receptors on MVN neurones at 4h following UL. Specifically, in the rostral ipsilateral MVN, there is a marked down-regulation of the efficacy of both $GABA_A$ and $GABA_B$ receptor function after UL, while in the contralateral MVN there is significant up-regulation of $GABA_B$ receptor function in both the rostral and caudal regions, and a marked up-regulation of $GABA_A$ receptor function in the caudal region.. It is probable that these changes in the efficacy of GABA receptors reported here are important in restoring the balance of activity between the vestibular nuclei *in vivo*. The decrease in GABAergic responsiveness of ipsilateral MVN cells will help to counteract the excessive commissural inhibition which these cells are subject to *in vivo*, and the increased responsiveness of contralateral MVN cells to GABAergic agonists will act to reduce the hyperactivity of these cells which follows UL, and therefore help to reduce the excessive inhibitory drive to the ipsilateral side.

It is interesting to note at this point, that while the changes in responsiveness to GABA agonists in the rostral region of the ipsilateral nucleus are accompanied by an

increase in the resting discharge rate, this is not the case with MVN cells located in any other region of either the ipsilateral or contralateral nucleus

Mechanisms of changes in GABA receptor efficacy in the MVN following UL

It is well known that neurotransmitter receptors are subject to regulation following their activation by agonists. The initial event in the regulatory pathway is a rapid (within seconds to minutes) desensitisation of receptors, producing a loss of immediate responsiveness. If agonist exposure persists, (for minutes to hours) receptors are then removed from the cell surface membrane and enter an internal membrane pool, a process referred to as internalisation or sequestration. The internalised receptors can either be recycled to the surface or degraded. Degradation is generally favoured by chronic (for hours to days) exposure to agonists and is often termed down-regulation (Klein et al., 1995).

It is unlikely that the changes in $GABA_A$ receptor efficacy observed in MVN neurones 4h post-UL is due to de-sensitisation, as the dose-response measurements were made after a 1h incubation period in normal aCSF, thus removing any excess GABA from the MVN slices which accumulated as a result of the excessive inhibition which these cells are subject to *in vivo*. Direct evidence showing a down-regulation of $GABA_A$ receptors after UL has been provided by Calza et al. (1992) who observed a decrease in benzodiazapine binding in the ipsilateral MVN within 3h post-UL, which fits well with the time-course of excitability changes observed in the first set of experiments described here. Thompson et al. (1986) described a significantly higher level of GABA immunoreactivity in the lesioned VNC 3 and 6 days after UL in the squirrel monkey. However, Li et al. (1996) showed that GABA levels were decreased in the early stages following UL but increased at later time points. The differences in this two studies may reflect the different animals used, but it is strange that the study by Li et al. (1996) found no significant left-right differences in GABA levels after UL, whereas Calza et al. (1992) and the present experiments described here clearly demonstrate a functional down-regulation of

GABA receptor efficacy following UL which occurs presumably in response to the excess inhibition which these cells are subject to *in vivo*.

It is conceivable that the changes in GABA receptor efficacy observed in the ipsilateral and contralateral MVN within 4h following UL persist indefinitely, so that the permanent loss of excitatory input from the ipsilateral primary vestibular afferents is, in effect, compensated for by a decrease in responsiveness of the ipsilateral MVN cells to inhibitory synaptic inputs. Further experiments to investigate the efficacy of GABAergic inhibition in the ipsilateral MVN at longer time points following UL are necessary to see if the rapid alterations in GABA receptor function are also involved in the longer-term maintenance of VC.

$GABA_A$ receptors

$GABA_A$ receptors are the major transducers of fast synaptic inhibition in the central nervous system, and have been shown to have distinct binding sites for GABA and modulators such as benzodiazepines (Chan et al., 1983), barbiturates (Study and Barker, 1981), and neurosteroids (Majewska et al., 1986), (for reviews see Burt and Kamatchi 1991; Rabow et al., 1995).

It is likely that the changes in $GABA_A$ receptor function following UL are due to the slower processes of internalisation of the activated receptors, or the down regulation of receptor affinity or efficacy, either through phosphorylation or alterations in the $GABA_A$ receptor subunit composition. The effects of chronic exposure of neurones to GABA and related agonists have been studied in several systems, mainly in neuronal cell-lines in culture (Malotreaux et al., 1987, Tehrani and Barnes, 1988). Prolonged incubation with GABA leads to a decrease in [3H]muscimol and [3H]flunitrazepam binding in cultures of embyronic cortical neurones (Malotreaux et al., 1987, Tehrani and Barnes, 1988). Although reductions in ligand binding do not necessarily imply a loss of function, these studies went on to show that in addition to loss of binding, neurones also showed reductions in GABA-dependent Cl^- flux and whole-cell patch-clamp studies demonstrated that the peak

amplitude of GABA-evoked currents was reduced by 60-70% (Hablitz et al., 1989). Calza et al. (1992) demonstrated that benzodiazepine binding significantly decreases within 3h post-UL in the ipsilateral MVN, suggesting that there is a reduction in the available receptors. Intracellular studies of MVN neurones after UL are now required in order to determine if the reduction in ligand binding in these neurones is correlated with a loss of receptor function, although, the marked changes in the time course of the inhibitory effects of muscimol on the firing rates of the MVN cells after UL in the present experiments suggest that there are significant changes in the binding kinetics and efficacy of the $GABA_A$ receptors.

More recent studies using reverse transcriptase-polymerase reaction (RT-PCR) have looked at the down-regulation of $GABA_A$ receptor subunit gene expression in cultured neurones in chick embryo cerebral cortex following chronic exposure to GABA (Baumgartner et al., 1994). These studies showed that a single dose of 100μM GABA added to the culture medium was sufficient to reduce the level of RNA for the α1, β1, β2 and γ1 subunits when analysed seven days after. If the medium was changed to a solution without added GABA, at four days, no significant decrease in the amount of the α1 mRNA was obtained (Baumgartner et al., 1994) even though a 50% reduction in [3H] flunitrazepam binding occurred at this time (Hablitz et al., 1989). These experiments suggest that the decline in transcript levels does not precede the reduction in binding, and that down-regulation of receptor mRNA is the final process which occurs only after prolonged inhibition to GABA. In the experiments presented in this thesis then, although we see a functional down-regulation of $GABA_A$ receptors in the ipsilateral rostral cells at 4hours post-UL, this is unlikely to be due to down-regulation of GABA receptor subunits and mRNAs and is more likely to be due to phosphorylation of ion channels and receptors a view which is supported by recent experiments by Sansom et al. (1997), who recently showed that MVN neurones following UL show changes in phosphorylation of several likely protein kinase C substrates. At present, little is known about the subunit composition of $GABA_A$ receptors in the MVN, currently we are using RT-PCR to resolve this situation. Indeed, it would be interesting to determine if there

any changes in subunit expression of $GABA_A$ receptor subunits following UL and at what time point these changes occur. If there are changes in $GABA_A$ receptor subunits, are these permanent? Answers to these questions would lead to a better understanding of the mechanisms involved in vestibular compensation.

$GABA_B$ receptors

$GABA_B$ receptors are coupled to guanine nucleotide binding proteins (G proteins) and modulate synaptic transmission through intracellular effector systems (Kaupmann et al., 1997; Bettler et al., 1998), hence the effects of activating $GABA_B$ receptors are generally longer lasting than those initiated by the $GABA_A$ receptors. Evidence suggests that both presynaptic and postsynaptic $GABA_B$ receptors exist (Bonanno et al., 1992, 1993; Lanza et al., 1993). Presynaptically, $GABA_B$ autoreceptors have been described controlling the release of GABA, whereas $GABA_B$ heteroreceptors regulate the release of glutamate, noradrenaline, dopamine, 5-hydroxytryptamine, substance P, cholecystokinin or somatostatin (for a review see Bowery, 1993). Postsynaptically, $GABA_B$ receptors are coupled to K^+ channels by a G-protein, thus $GABA_B$ receptor activation results in an increase in K^+ conductance and a resulting hyperpolarisation of cells.

To date, there are few examples of $GABA_B$ receptor down regulation. $GABA_B$ receptors have been shown to be down-regulated in the spinal cord dorsal horn following 21 days of intraperitoneally administration of the $GABA_B$ agonist, baclofen, (Malcangio et al., 1993), or 3-4 weeks following lesion of the sciatic nerve (Castro-Lopes et al., 1995). This pattern of $GABA_B$ down-regulation was also observed 17 days after a lesion of the sciatic nerve in the ipsilateral dorsal root ganglia (Towers et al., 1997). In the present experiments a rapid change in $GABA_B$ receptor efficacy was observed in both the ipsilateral and contralateral MVN 4h after UL, this may be the first demonstration of such a rapid change in the efficacy of $GABA_B$ receptors.

$GABA_B$ receptors may be modulated not only at the level of the membrane receptor itself but also by modulation of the G-protein coupled second-messenger

systems downstream of the receptor. G-proteins are subject to the same modes of regulation as ligand gated ion channels, namely, desensitisation, sequestration and down regulation (Carman and Benovic, 1998). Desensitisation of G-proteins is primarily mediated by kinases, such as protein kinase A (PKA) and protein kinase C (PKC), that are responsive to second messengers and also by G-protein coupled receptor kinases (GPRK) which specifically phosphorylate activated G-protein receptors (for review see, Krupnick and Benovic, 1998). GPRKs are in turn, themselves regulated (Stoffel et al., 1997), and it is conceivable that many regulatory mechanisms are involved in the desensitisation and subsequent down regulation of $GABA_B$ receptors.

Rapid changes in functional down-regulation of $GABA_A$ and $GABA_B$ receptors

The rapid changes observed in both $GABA_A$ and $GABA_B$ receptor efficacy within 4h after UL, may then present a novel system in which to study the intracellular signalling pathways which are involved in the functional down regulation of both receptor subtypes.

Other mechanisms involved in the increase in excitability of rostral ipsilateral MVN neurones

Changes in GABA receptor efficacy were observed in all regions of both nuclei at 4h post-UL, however these changes were not all accompanied by changes in the mean resting discharge rate. Only MVN neurones in the lesioned MVN showed a significant change in mean resting discharge rate following UL. It is likely then, that other, currently unidentified, mechanisms are important in generating the increase in mean discharge rate seen in these cells 4h after surgery. MVN neurones are known to possess intrinsic pacemaker-like conductances that generate a resting discharge independent of synaptic input from the VIIIth nerve afferents (Gallagher et al., 1985, 1992; Darlington et al., 1989; Dutia et al., 1992). For example, MVN neurones are known to possess calcium -activated potassium currents which are involved in the AHP, a down-regulation of these conductances would lead to an increase in

excitability of these cells. An up-regulation of the persistent sodium conductance which these cells have also been shown to possess would also result in an increase in excitability as seen in the present experiments. Intracellular studies are now required in order to determine if there are changes in the membrane conductances of rostral lesioned MVN cells.

Summary

The present results demonstrate that a significant increase in the excitability of lesioned rostral MVN cells develops within 2-4h post-UL and lasts for up to 1 week. This significant increase in excitability is likely to be important in restoring the resting discharge rate of MVN cells following UL *in vivo* during the initial stage of VC. This increase in the excitability of ipsilateral rostral MVN cells will serve to counteract and overcome the initial disfacilitation due to the loss of the primary afferent input and the increased commissural inhibition from the hyperactive contralateral MVN neurones and Purkinje cells from the vestibulocerebellum.

The changes in GABA receptor efficacy observed in these experiments thus indicates that a key process in the early behavioural recovery after UL is the reduction of the imbalance in commissural and cerebellar inhibition that immediately follows the lesion through the dynamic adjustment, over a period of hours, of GABAergic synaptic efficacy on the lesioned and intact side. Furthermore, this system may become a powerful tool for studying the intracellular signalling pathways which are involved in the rapid functional down-regulation of both $GABA_A$ and $GABA_B$ receptor subtypes *in vivo*.

Although it is likely that the changes in GABA receptor efficacy are functionally compensatory, this may not be the only mechanism involved in generating the returning resting discharge rate to ipsilateral MVN neurones following UL. Changes in GABA receptor efficacy were observed in all regions of both nuclei at 4h post-UL, however these changes were not accompanied by changes in the mean resting discharge rate. Only ipsilateral rostral MVN neurones showed a significant change in mean resting discharge rate following UL. It is likely then, that other, currently unidentified mechanisms are important in restoring the mean resting discharge rate after UL.

STRESS AND NEURAL PLASTICITY IN MVN NEURONES

4.1 INTRODUCTION

During stress, the glucocorticoid steroids, (cortisol in man, and corticosterone in the rat), are secreted by the adrenal cortex. Their synthesis and release are under the control of adrenocorticotrophic hormone (ACTH), which is secreted from the anterior pituitary gland. Glucocorticoids have significant anti-inflammatory and immunosuppressive activity for which they are often used therapeutically (see Kawata, 1995; Wilkens, 1995; Brann *et al.*, 1995, for a review). The process of vestibular compensation is sensitive to stressful events. For example, an abrupt movement by the observer can cause spontaneous nystagmus to return to a compensating guinea pig and in well compensated humans decompensation may occur as the result of fatigue (Katsarkas and Segal, 1988). Glucocorticoids are currently used in vestibular related disorders. Short-term treatment with methylprednisolone has been reported to reduce vertigo due to conditions such as peripheral vestibular neuritis (Ariyasu *et al.*, 1990). Jerram *et al.* (1995) reported that systemic administration of methylprednisolone before, and 4h post-UL, reduced the frequency of spontaneous nystagmus (SN). Yamanaka *et al.* (1995) showed that administration of the synthetic glucocorticoid, dexamethasone, prior to surgery facilitates the behavioural recovery following UL in the rabbit, in contrast, administration of the glucocorticoid antagonist, RU38486, delayed compensation. However, in a recent study by Alice *et al.* (1998) systemic administration of dexamethasone was found to have no effect on the rate of compensation of SN in the guinea pig. Recently, immunocytochemical studies in the rat have shown that a moderate density of the type II glucocorticoid receptor (GR) exists in the MVN and low densities are present in the SVN and LVN (Ahima and Harlan, 1990), suggesting that glucocorticoids may have a direct action on MVN neurones. This is a view which is supported by the work of Yamanaka *et al.* (1995) who demonstrated that iontophoretic administration of dexamethasone to the VNC produced a dose dependent increase in the firing rate of type I MVN neurones, which could be blocked by the glucocorticoid antagonist, RU38486.

Together these studies suggest that glucocorticoids may have an important role in the process of VC.

4.2 LITERATURE REVIEW

4.2.1. The stress response

Mammals have developed a complex system in order to maintain homeostasis, both under resting and stressful conditions. It is important to distinguish between long-term stress and short-term stress. During short-term stress, often referred to as "alarm" or "fright", minute-to-minute changes in metabolism are under the control of catecholamine hormones, primarily adrenaline, whose secretion is controlled by the autonomic nervous system. Short-term stressors are events of surprising nature whereas long-term stressors include events like intensive cold, serious injury, burns, and surgery.

The hypothalamo-pituitary-adrenocortical (HPA) axis originates in neurones containing corticotrophin-releasing hormone (CRH) in the hypothalamic paraventricular nucleus (PVN), which send their axons to the external zone of the median eminence (Chrousos, 1992). In response to a variety of stimuli CRH is released into the portal circulation stimulating adrenocorticotrophin hormone (ACTH) release. Activation of the HPA axis has been shown to be stimulated by noradrenaline (Aghajanian and Van Der Maelen, 1982), serotonin (Bagdy *et al.*, 1989), acetylcholine (Calergo *et al.*, 1988), and histamine (Kjaer *et al.*, 1994), and inhibitied by GABA (Calogero *et al.*, 1988), the opioid peptides (Nikolarakis *et al.*, 1986), and the glucocorticoids (Feldman and Weidenfeld, 1995). The parvocellular neurones of the PVN, innervate and are innervated by the opioid peptide producing neurones of the arcuate nucleus of the hypothalamus. Therefore, activation of the stress system stimulates opioid peptide secretion, which reciprocally inhibits the activity of the stress system and also, through projections to the hindbrain and spinal cord, produces analgesia (Nikolarakis *et al.*, 1986).

Although during stress, CRH is the primary mediator of ACTH release, other hypothalamic hormones, such as vasopressin and oxytocin, can also contribute to this process (Swanson *et al.*, 1983; Antoni, 1986; Plotsky, 1991; Whitnall, 1993). Release of these hormones from the hypothalamus induces ACTH secretion from the adrenal glands. ACTH molecules bind to specific receptors on the outer cell membrane of all

three layers of cells of the adrenal cortex. The role of ACTH is to produce biochemical changes that increase the level of cholesterol. Once produced cholesterol is metabolised to produce both glucocorticoids (GC), and mineralcorticoids (MR). Most of the secreted hormone is transported to its target bound to a corticosteroid-binding protein, transcortin. Only a small amount travels as free hormone. The glucocorticoids exert a tonic inhibitory effect on CRF and ACTH secretion, as evidenced by their elevation following bilateral adrenalectomy. GC also have an inhibitory effect on stress-induced elevations of plasma ACTH and corticosteroids following a variety of stressful stimuli (Keller-Wood and Dallmann, 1984).

4.2.2 Glucocorticoid actions

Glucocorticoids act by binding to two types of intracellular receptor, the glucocorticoid (GR) and the mineralcorticoid (MR) receptors. Both receptors have recently been characterised by binding studies and more recently by isolation and sequencing of cDNA clones, which correspond to the genes of the two receptors (reviewed in, De Kloet, 1991). Brain MR receptors are expressed largely by neurones in the limbic regions and to a lesser extent in some thalamic nuclei, brainstem reticular formation, sensory and motor nuclei, cerebellum and spinal cord (Arriza *et al.*, 1988; Funder and Sheppard, 1987; McEwen *et al.*, 1986; Reul and DeKloet, 1985; Reul *et al.*, 1989). The MR receptor has a high affinity for corticosterone and aldosterone, the principal glucocorticoid and mineralcorticoid, respectively, in rats (Funder and Sheppard, 1987; McEwen *et al.*, 1986), and is hence largely occupied under basal conditions. GR receptors are expressed widely in the CNS by neurones and glia. High levels of the receptor are expressed in the hippocampus, neocortex, cerebellum, thalamus and stress-related nuclei of the hypothalamus and brainstem (Funder and Sheppard, 1987; Reul and DeKloet, 1985; Reul *et al.*, 1989; Arronson *et al.*, 1988; Ahima and Harlan, 1990), and have an approximately 10 fold lower affinity for corticosterone compared to MR receptors. It is the GR receptor which is believed to mediate the effects of stressful stimuli (Reul *et al* 1987, Spencer *et al* 1990).

Much has become known about the properties (Evans, 1988; Fuller, 1991; Gronenmeyer, 1992; Trapp, 1994) and distribution of intracellular steroid hormone receptors in the past ten years (Ahima and Harlan, 1990; Ahima *et al.*, 1991). Two types of action have been demonstrated to be mediated by steroid hormones. Classical actions which act via intracellular steroid receptors and are usually slow in onset and prolonged in duration, and more recently, rapidly evoked changes in electrical properties of cells have been demonstrated both by steroids and neurosteroids (for review see, Joëls, 1997; Zakon, 1998).

Genomic actions

Steroids are membrane soluble and once inside the cytoplasm of the cell, glucocorticoids combine with the specific, high-affinity receptor to form a steroid-receptor complex. The steroid-receptor complex undergoes conformational changes that enables it to translocate to the cell nucleus, where it binds to specific hormone response elements in the genome to enhance or suppress transcription (Katzenellenbogen *et al.*, 1996). To study the delayed effects of steroids, neurones have to be examined for several hours, because the genomic actions take at least 30 min to develop. Thus recordings from neurones are not always feasible, even when attempted *in vitro*. As an alternative, neuronal activity before steroid application is compared with that several hours after the steroid is administered (Joëls, 1997). Many studies of steroidal action have been carried out on the hippocampal brain slice, since hippocampal CA1 pyramidal neurones express a high density of both MR and GR receptors (Van Steensel *et al.*, 1994). With the use of selective ligands for MRs and/or GRs, discrimination between the physiological effects mediated by either of these receptor types is possible.

Depolarization of hippocampal neurones induces a brief burst of action potentials, after which the cell ceases to fire. This attenuation (accommodation) of firing activity is due to activation of Ca-dependent K conductances which underlie the AHPs of these cells (Gustaffson and Wigstrom, 1981; Lancaster and Adams, 1986). Both the accommodation of firing and AHP serve to reduce the excitability of the pyramidal neurone. It has been demonstrated that the slow AHP following a burst of action

potentials is modulated by corticosteroid hormones, such that, occupation of MRs reduces the accommodation and the amplitude of the AHP (Beck *et al.*, 1994; Joëls and de Kloet, 1990). By contrast, occupation of GRs enhances accommodation and the amplitude and duration of the slow AHP (Joëls and de Kloet, 1989; Kerr *et al.*, 1989). The steroid effects on firing behaviour developed over the course of approximately 1h and persisted for the duration of the *in vitro* experiment, thus suggesting a gene-mediated action. Corticosterone has also been shown to upregulate delayed rectifier and A-type K currents, and decrease the low-voltage activated Ca current which lead to a decrease in membrane excitability (Levitan *et al.*, 1991; Meza *et al.*, 1994).

Rapid membrane action

Fast actions by corticosteroid hormones have been observed mainly in three areas of the brain: the hypothalamus, hippocampus and the brainstem. In the PVN of the hypothalamus, neurones are mostly inhibited by cortisol and corticosterone (Chen *et al.*, 1994; Kasai and Yamashita, 1988; Saphier and Feldman, 1988). Inhibitory effects by corticosteriod hormones were observed both *in vivo* and in hypothalamic slices (Chen *et al.*, 1994), indicating that local steroid actions suffice to induce these inhibitory actions. These inhibitory effects could be antagonised by the glucocorticoid antagonist, RU38486. In the hippocampus, corticosteroid hormones also tend to suppress neuronal excitability. Synaptic responses in the hippocampus which are mediated by glutamate receptors are clearly modulated by corticosteriod hormones. The onset of these steroidal effects is usually within 10-20 min after application and they have been shown to persist for at least 1 h. No evidence that these actions are mediated via a genomic action has been forthcoming.

In the brainstem the action of corticosteriod hormones appears to be predominantly excitatory. Both with peripheral, and ionotophoretic application of corticosterone single unit firing was increased in reticular formation and dorsal raphe neurones (Avanzino *et al.*, 1983; 1984; Dubrovsky *et al.*, 1985). The effects were rapid, occurring within two minutes after ionotophoretic application (Avanzino *et al.*, 1983), and of short duration, supporting the involvement of a membrane receptor for the steroids.

Actions of neurosteroids

It has become clear in recent years that some of the metabolites of peripherally released steroids and brain-derived neurosteroids can evoke rapid changes in electrical properties of neurones (Carette and Poulain 1984; Joëls, 1997). These neurosteroids rapidly alter the excitability of neurones by binding to membrane-bound receptors for neurotransmitters, such as glutamate and GABA (Paul and Purdy, 1992), and not via genomic effects mediated through intracellular or nuclear receptors. For example, dehydroepiandosterone (DHEA), and its sulfatide-sulfate derivatives are secreted in considerable amounts, from the adrenal cortex during stress (Corpechot *et al.*, 1981), and have been shown to act as non-competitive modulators of the $GABA_A$ receptor (Demirgoren *et al.*, 1991). Low doses of other neurosteroids, pregnenolone, pregnenolone sulphate, allotetrahydrocorticosterone and pregnanolone have been shown to rapidly and reversibly, suppress voltage-gated N- and L-type Ca currents in dissociated hippocampal cells (ffrench-Mullen and Spence, 1991; Spence *et al.*, 1991).

Ligand-gated Ca flux has also been shown to be affected by neurosteroids. Pregnenolone sulphate specifically enhanced NMDA-receptor currents in cultured spinal cord chick neurones (Wu *et al.*, 1991). The most intensively investigated effect of neurosteroids concerns their interaction with the $GABA_A$ receptor complex. Allopregnanolone rapidly potentiated the inhibitory responses of cerebellar Purkinje neurones to GABA when recorded extracellularly *in vivo* (Smith *et al.*, 1987). More recently, Yamamoto *et al.* (1998) demonstrated that the neurosteroid, dehydroepiandrosterone sulphate, (DHEAS) when applied by microphoresis to MVN neurones *in vivo*, had no effect on the spontaneous and rotation induced firing of these cells. However, DHEAS dose-dependently blocked the decrease in firing which occurs when GABA is applied to MVN neurones. This effect occurred within a few seconds after administration of the neurosteroid suggesting a membrane action.

Combined actions

It is possible that both genomic and membrane-bound actions of steroids occur at the same cell. Long-term exposure to a steroid, presumably acting through genomic

mechanisms may upregulate membrane receptors for the same or other steroids (Tischkau and Ramirez, 1993). Given that prolonged activation of second messenger pathways can result in changes in gene expression (McEwen, 1996), it is likely that membrane receptors also activate transcription in some situations. In some cells, responses could depend on simultaneous activation of both pathways.

4.2.3 Immediate early genes

Genes that respond to transynaptic stimulation and changes in membrane potential can be categorised into two major classes: genes that are rapidly and transiently expressed within minutes of stimulation, the immediate early genes (IEGs; Greenberg and Ziff 1984, for review see Hughes and Dragunow 1995), and genes that are induced or repressed slowly, over a few hours and whose expression tends to require *de novo* protein synthesis, the late response genes (LRGs; Klarsfield *et al.*, 1989). It has been proposed that IEGs encode for proteins that can control the expression of late response genes whose expression alters neuronal function (for review see Sheng and Greenberg, 1990).

The first immediate early genes identified, *c-fos* and *c-myc* were identified by their induction in cell lines after a brief exposure to growth factor (Kelly *et al.*, 1993; Greenberg and Ziff, 1984). The initial experiments were carried out in *in vitro* cell lines but the induction of *c-fos* was subsequently demonstrated *in vivo* in response to seizures (Morgan *et al.*, 1987). Alterations of external and internal environments cause rapid changes in gene expression in the brain. For example, when animals are restrained, proto-oncogenes such as *c-fos* are activated immediately in discrete brain regions (Ceccatelli *et al.*, 1989; Stone and Zhang, 1995). Both *c-fos* and *c-jun* are the most established and investigated of the IEGs and the protein products of these IEGs, Fos and Jun have been used by many researchers as markers of cellular activation in a variety of *in vitro* and *in vivo* studies (Morgan and Curran, 1986).

Transcription and activation of the Fos gene occurs within 5 min and continues for 15-20 minutes (Greenberg and Ziff 1984; Greenberg *et al.*, 1985). The mRNA accumulates and peak values occur at 30-45min after stimulation (Muller *et al.*, 1984), after which it declines with a relatively short half life of about 12 min. Synthesis of the Fos protein follows the synthesis of the mRNA with a half life of about 2 hours (Muller *et al.*, 1984, Curran *et al.*, 1984), and peaks at 60-90 min Sharp *et al.*, 1991). The protein product of *c-fos* has been found to act as an autorepressor of its own transcription (Wilson and Treisman 1988), which probably accounts for its rapid and transient induction. (Sassone-Corsi *et al.*, 1988; Guis *et al.*, 1990).

Expression of IEGs in response to a stimulus therefore indicates the following; one, the cell has actually been activated by the stimulus, and two, the cell has initiated the modulation of expression necessary for a particular cellular response (Senba and Ueyama, 1997).

Induction of IEG expression

Neuronal gene expression can be modulated by neurotransmitters, membrane electrical activity and neurotrophic growth factors (Sheng and Greenberg, 1990). Extracellular stimuli cause receptor-mediated activation of second messengers and Ca^{2+} influx which results in the activation of various protein kinases (Sheng and Greenberg, 1990; Karin, 1995). It has been suggested that this rise in intracellular Ca^{2+} leads to the increase in *c-fos* expression. Activation of Ca^{2+} permeable NMDA glutamate receptors in cultured cerebellar granule cells resulted in increased *c-fos* expression (Szekely *et al.*, 1987). In PC12 cell lines, nicotinic receptor activation which leads to increased intracellular Ca^{2+} levels, induced c-fos expression (Greenberg *et al.*,1986). Together these results suggest that increased intracellular Ca^{2+} levels leads to the activation of cellular mechanisms that then regulate the expression of IEGS. However, growth factor mediated induction of *c-fos* gene expression in PC12 cells does not require Ca^{2+} influx (Morgan and Curran, 1986). Instead this pathway seems to involve protein kinase C (PKC) activation, in that the induction of *c-fos* in adipocytes is reduced by phorbol-ester pre-treatment of cells, which depletes cellular PKC activity (Stumpo and Blackshear, 1986).

Together these early studies suggest that at least two different signalling pathways activate *c-fos* expression, the first involving the activation of the inositol-phosphate-PKC pathway, the second involving an increase in intracellular Ca^{2+} ions.

IEGs and the control of gene expression

It has been demonstrated that the protein products of *c-fos* and *c-jun* interact with each other to form a heterodimeric transcription factor complex (Halazonetis *et al.*, 1988; Kouzarides and Ziff 1988;) through a conserved dimerized domain known as the

"leucine zipper" through their side chains (Kouzarides and Ziff, 1988). A Fos-Jun complex is referred to as AP-1 and binds to AP-1 binding sites of target genes (Rauscher *et al.*, 1988). Genes for precursor peptides of enkephalin (Sonnenberg *et al.*, 1989), dynorphin (Naranjo *et al.*, 1991), somatostatin (Kovaxix-Milivojevic and Gardner, 1992), and tyrosine hydroxylase (Gizang-Ginsberg and Ziff, 1990) are considered to be target genes of AP-1, although the target genes of AP-1 in neurones activated by stress stimuli have yet to be determined. By binding to *c-jun*, Fos directly modulates *c-jun* transcriptional ability by forming a heterodimer of Fos and Jun proteins (Kouzarides and Ziff, 1988). Although *c-jun* proteins can form homodimers (c-Jun/c-Jun) that bind to, and weakly transactivate, gene expression from AP-1 sites, Fos proteins do not form homodimers and hence can not bind to the AP-1 sites to transactivate gene expression (Hughes and Dragunow, 1995).

It has recently been shown that ligand activated transcription factors, for example, the glucocorticoid and thyroid hormone receptors, also interact with Fos/Jun complexes to modulate their transcactivational ability (Lucibello *et al.*, 1990; Lopez *et al.*, 1993; Schmidt *et al.*, 1993).

IEGs and stress

The first application of the IEG, *c-fos* to stress studies was made by Ceccatelli *et al.* (1989). When animals were subjected to stressors, such as restraint, mRNAs of the *c-fos* and *c-jun* family genes were expressed immediately (within a few minutes) in the hypothalamus, pituitary and adrneal glands (the HPA axis) (Senba *et al.*, 1994). Some of the IEGs, such as *c-jun*, *jun D* or *zif268*, are constitutively expressed in certain brain areas, however, others, such as *c-fos* are expressed transiently, peaking after 30 to 60 minutes and then declining (Senba *et al.*, 1994). Other models of stress, for example, swim stress (Cullinan *et al.*, 1995), foot shock (Li *et al.*, 1996), and peripheral inflammation (Wan *et al.*, 1994), have also demonstrated similar expression of Fos immunoreactivity in the HPA axis.

Use of IEG to investigate the molecular mechanisms of VC

Studies on the expression of immediate early genes (IEG), such as *c-fos*, can be useful in understanding the molecular mechanisms which may underlie the process of vestibular compensation. Fos, the protein product of the IEG *c-fos*, has been used as a marker of cellular activation to determine areas of the CNS which may be activated after unilateral labyrinthectomy in a number of previous studies (Kaufman *et al.*, 1992; Kaufman *et al.*, 1993; Kitahara *et al.*, 1995; Cirelli *et al.*, 1996; Darlington *et al.*,1996; Sato *et al.*, 1997). These studies have shown that Fos immuno-positive cells appear between 2 and 3 hours after UL in the ipsilateral and contralateral MVN, the contralateral beta subnucleus of the inferior olive, and both the flocculus and paraflocculus of the cerebellum. All of these areas may have an important role in VC (see section 2.5 for a discussion). Some disagreement exists as to which of the two MVN express Fos immunoreactivity. In the studies by Kitahara *et al.* (1995) and Kaufman *et al.* (1992) both groups demonstrated increased Fos immunoreactivity in the MVN ipsilateral to the lesion. In the study by Cirelli *et al.* (1996) significantly higher levels of *c-fos* mRNA and Fos protein were found in the contralateral MVN between 3 and 6 hours post-UL. Darlington *et al.* (1996) described a bilateral increase of Fos immunoreactivity. The reasons for such differences are not clear. Species differences may account for the differences in the studies of Darlington *et al.* (1996) as they used guinea-pigs compared to the use of rats in the other studies. It is difficult however, to explain the differences in the results obtained by Kitahara *et al.* (1995), and Cirelli *et al.* (1996), as both groups used the same strain of animals (male wistar rats).

Two studies have examined the changes in both Fos and Jun expression in the brainstem after UL. These studies are more specific in that, it is known that in order to bind to AP-1 binding sites of target genes Fos proteins must form a complex with Jun proteins (Kouzarides and Ziff, 1988). Kitahara *et al.* (1995) demonstrated the presence of Fos immuno-positive cells in the ipsilateral MVN 1h after UL, but did not detect any Jun-immunoreactivity in any of the vestibular nuclei at any time point (1h-28days post-UL). In a more recent study, Darlington *et al.* (1996) demonstrated

the presence of both Fos and Jun proteins in the MVN, Fos immunoreactivity was found to increase immediately after UL whereas Jun expression increased later. This study therefore suggests that neuronal activation does occur in these neurones.

Other compounds released in response to stressful stimuli

Although increased levels of CRH and ACTH ultimately result in glucocorticoid release from the adrenal cortex both these compounds have been shown to have possible roles in the vestibular system.

1. Corticotrophin-releasing factor (CRF)

CRF, a 41 amino acid peptide plays a role in co-ordinating the overall response of the body to stressors as has been discussed in section 4.3. However, CRF has also been implicated as a potential cerebellar transmitter or modulator (Errico and Barmack, 1993). CRF has been found in the neurones of the inferior olive, which is the exclusive origin of cerebellar climbing fibres (Young *et al.*, 1986; Cummings *et al.*, 1988, 1989; Barmack and Young, 1990; Yamano and Tohyama, 1994). CRF-immunoreactive mossy fibres have been shown to originate from the MVN, and the nucleus prepositus hypoglossi (Errico and Barmack, 1993). Ligand binding studies with radiolabeled CRF have demonstrated the presence of specific CRF receptors in the molecular layer of the cerebellum where CRF-immunoreactive climbing fibres terminate and in the cerebellar granule cell layer where CRF immunoreactive fibres terminate (DeSouza *et al.*, 1985; DeSouza, 1987; Chai *et al.*, 1990;). These observations suggest a role for CRF in central neuronal transmission. The role of CRF in VC has been studied. Kaufman *et al.* (1994) demonstrated that the level of CRF mRNA was significantly increased in the contralateral beta subnucleus of the inferior olive, which has been shown to have a projection to the uvula and nodulus of the vestibulocerebellum (Kaufman *et al.*, 1996). It is possible then that there is an increase in CRF levels following UL, due to the activation of the stress axis, and this compound may have other, as yet unidentified, roles in VC.

2. Adrenocorticotropic hormone (ACTH)

Melanocortins are a family of peptides, containing the sequence ACTH(1-13). However, these compounds are devoid of corticotrophic activity (Darlington *et al.*, 1996). Short fragments of ACTH, ACTH (4-10), ACTH (4-7), ACTH (1-10) or ACTH (1-13) have been shown to enhance compensation in the frog, (Flohr and Luneberg, 1982; 1989), whereas longer ACTH fragments (ACTH(1-24) and ACTH(1-24)), and ACTH fragments (ACTH (4-6) and ACTH(5-7)), had no effect (Flohr and Luneberg, 1982; 1989). In the guinea pig (Gilchrist *et al.*, 1990; 1994), and squirrel monkey (Igarashi et al., 1985, 1988), administration of ACTH (4-10) was found to enhance the ocular motor symptoms produced by vestibular deafferentation. At present, it is unknown whether this particular fragment of ACTH is found in the brains of UL treated animals.

4.3 AIMS

The following experiments were designed to investigate the role of the stress axis in generating the compensatory increase in excitability of lesioned rostral MVN neurones. As shown in chapter 3, there is a significant, sustained increase in the excitability of rostral ipsilateral MVN neurones within 4 hours after UL and this provides a good model in which to investigate the role which the stress axis may play in vestibular compensation.

1. In order to investigate the effect of endogenous stress hormones on the increase in excitability of MVN neurones *in vitro*, 12 animals received a left UL under urethane anaesthesia (ureth+UL) and were kept in a temperature regulated cage for either 4h (n=6), or 6h (n=6), following surgery. These animals therefore did not wake up at any time after the UL and thus exhibited none of the behavioural symptoms of UL and experienced none of the stress normally associated with the vestibular deafferentation syndrome.

2. In order to determine that the firing rate of MVN neurones was not affected by the anaesthetic used, 4 animals received a sham-operation under urethane anaesthesia (ureth+sham), and were then kept in a temperature regulated cage for 4h after the surgical procedure.

3. To mimic the endogenous activation of the stress axis which would normally occur in alert labyrinthectomised animals, the synthetic glucocorticoid receptor agonist, dexamethasone, was administered (5mg/kg), 30 min before the lesion and 2h post lesion to urethane anaesthetised labyrinthectomised animals (ureth + UL + dex) (n=5). To control for any direct effects of dexamethasone on the firing rate of MVN neurones, 5 sham-operated urethane anaesthetised animals received the same protocol of dexamethasone administration (ureth+ sham +dex).

4. In the converse set of experiments animals were allowed to awaken after receiving a left UL under Avertin anaesthesia as described in the previous chapter (section3.2). To block the activation of endogenously released stress hormones, animals were given either an intra-muscular (i.m.), injection of the glucocorticoid antagonist, RU38486, (5mg/kg, n=4), or an intraperitoneal (i.p.), injection of the mineralcorticoid antagonist, spironolactone, (5mg/kg, n=4), at the time of surgery.

5. To investigate the effects of glucocorticoid agents on the rate of VC, circular walking was used as an index of behavioural recovery following UL. 12 animals received a left UL and an I.P. injection of saline 30 min before the induction of anaesthesia and 2h after. The frequency of circular walking to the ipsilateral side was then recorded every hour for a total of 7 hours after UL (see section 3.2)

In groups of 12 again, animals were either treated with the glucocorticoid agonist dexamethasone (5mg/kg i.p.) or the glucocorticoid antagonist, RU8486, (5mg/kg i.m.) before UL and the incidence of circular walking was recorded for a total of 7 hours post-UL (see section 3.2).

6. Finally, to investigate the activation of the stress axis in animals which were allowed to recover following surgery. Animals either received a sham operation or a left UL under Avertin anaesthesia, they were then allowed to awake and experience the stress associated with the surgery. After a time period of either 1.5 (n=12), 3 (n=9) or 6h (n=10), the animals were anaesthetised under halothane and the brains removed and prepared for Fos immunohistochemistry (see methods, section 4.4)

4.4 METHODS

Except for the following, the methods employed in chapter 4 of this thesis are identical to those described in the methods section of chapter 3. All values in this section are given as the mean ± s.e.m.

Use of urethane for non-recovery electrophysiology experiments

For non-recovery experiments rats were given a single (i.p.) injection of urethane (ethyl carbamate, 25% weight/volume solution; Sigma, UK) at a dose of 1.25g/kg, which produced the required level of anaesthesia for the duration of the experiments.

Immunohistochemistry

1. Tissue Recovery

Rats were anaesthetised using halothane, decapitated and their brains rapidly removed and deep frozen by plunging them into 2-methylbutane cooled to minus 30°C. 15 micron coronal sections were cut on a cryostat (Bright Instruments, Huntington, England) at the level of the paraventricular nucleus (PVN), and thaw-mounted on gelatin-coated slides. The slides were then stored in slide boxes and kept at -70 ^{0}C until processed.

2. Methodology

Day1

Slide boxes were removed from the freezer and allowed to come up to room temperature for 1h. Slides were removed and placed into glass slide boxes for immunohistochemisty.

Sections were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde for 30 min. The slides were then washed in 0.2% Triton X-100 in 0.1M PB for 4 x 15 min, the solution was replaced with the methanol solution for 20 min to deactivate endogenous peroxidase. The methanol was washed off with 0.2% Triton X-100 in 0.1M PB for 3 x 10 min. Triton X-100 is a detergent which increases tissue impregnation. Background binding was then reduced by washing with preincubation buffer containing 1% normal

goat serum for 30 min at room temperature. After drying off excess liquid from around the sections, slides were placed in flat slide boxes containing filter paper soaked in 0.1M PB. Approximately 50µl (an excess) of incubation medium containing the primary antibody Fos, was pipetted onto each test section. The slide boxes were covered to maintain humidity and slides were incubated for 24h at 4^0C. Additional antibody solution was added early the next day, to prevent the sections from drying out.

Day 2

Slides were washed with 0.2% Triton X-100 in 0.1M PB for 10 min to remove excess antibody. Excess liquid was tipped off the slide and the area around the sections was dried and slides then replaced in the flat slide boxes. Excess secondary antibody, was applied to test sections and incubated for 30 min. Slides were then washed with 0.2% Triton X-100 in 0.1M PB for10 min to remove excess secondary antibody. Excess liquid was tipped off the slide and the area around the sections was dried and slides then replaced in the flat slide boxes. The ABC reagent was freshly made up before use and slides were incubated in this solution for 1h. The ABC kit contains Avidin DH and biotinylated horseradish peroxidase H reagents which have been specially prepared to form ideal complexes for immunoperoxidase staining. Again, the slides were washed with 0.2% Triton X-100 in 0.1M PB for 10 min and dried as explained previously. Slides were then incubated in the peroxidase substrate solution (VIP) for approximately 10 min. Progress of the reaction was followed by viewing in a light microscope. Once the reaction worked and some nuclei appeared purple the reaction was stopped by washing the slides in distilled H_2O for 5 min. Slides were then mounted and coverslipped.

5. Quantitative Analysis

The presence of Fos immunoreactivity was detected by brightfield microscopy as a purple reaction product in the cell nuclei. For each animal, 6 sections (between Bregma -0.92mm and -1.8mm) were examined, (Kyowa optical unilux microscope, magnification x40) and Fos-positive cells within the Paraventricular nucleus (PVN)

counted with the aid of a grid, on both the left and right sides of each section examined. The same sections were then examined using an 'Improvision' image analysis system utilizing 'image' software, to measure the area of the PVN. The number of fos-positive cells/mm^2 for each section was calculated, and from this the mean of fos-positive cells/mm^2 for each animal. and each animal group obtained ± s.e.m. Statistical analysis was performed using Students t-test.

4.5 RESULTS.

Extracellular recordings from tonically active MVN cells were made in brainstem slices prepared from animals which had received either a left UL or a sham procedure. All MVN cells included in the study showed a regular sustained discharge for 90s. Each MVN was divided into three approximately equal parts, namely rostral, middle and caudal and the rostro-caudal location of each cell was recorded. The mean discharge rates of MVN cells located in the rostral and caudal thirds of treated animals was then compared with its corresponding control group. As only changes in the rostral third of the ipsilateral nucleus had been shown to occur in the previous experiments (see section 3.3), in these subsequent studies only the mean discharge rates of cells in the rostral and caudal thirds were compared.

As demonstrated in section 3.3 and shown in figure 4.1 the mean discharge rate of cells in the rostral MVN of the lesioned nucleus in slices prepared from alert animals 4h following UL (21.68 ± 1.6 spikes/s, n=30 cells), were significantly higher than that in slices prepared from sham-operated animals (15.16 ± 0.9 spikes/s, n=83 cells, $p<0.001$, MWRST). This confirms the result of the first series of experiments described in section 3.3, where a significant increase in the excitability of lesioned rostral MVN cells was observed after UL.

Effect of urethane anaesthesia on mean resting discharge rate of MVN cells *in vitro*

As shown in figure 4.2 in animals which received a sham procedure and remained anaesthetised (ureth + sham) for the following 4h, there was no difference observed in the mean discharge rate of MVN cells in either the rostral (ipsilateral MVN: 14.86 ± 1.45 spikes/s, n=35 cells, contralateral MVN: 13.77 ± 1.24 spikes/s, n=35 cells Table 4.1) or caudal regions (ipsilateral MVN: 11.89 ± 1.47 spikes/s, n=18 cells, contralateral MVN: 13.15 ± 2.43 spikes/s, n=15 cells Table 4.1.). These values did not differ from those obtained from sham-operated animals which were allowed to recover following surgery (Table 3.1a), and therefore suggest that the anaesthetic is having no effect on the mean discharge rate of MVN neurones.

In MVN slices prepared from animals which underwent a surgical labyrinthectomy but remained deeply anaesthetised for the 4-6h period following UL (ureth + UL), cells recorded from the rostral portion of the lesioned MVN showed a slight, but not significant, increase in their mean discharge rate (16.79 ± 1.49 spikes/s, n= 58 cells at 4h, 16.98 ± 1.2 spikes/s, n=61cells at 6h, figure 4.2. Table 4.1), when compared to cells recorded from the nuclei of ureth + sham animals (14.31 ± 0.95 spikes/s n =70 cells, fig 4.1). Thus the labyrinthectomy alone is not sufficient to induce the compensatory increase in excitability in MVN cells, as it does not occur if the animals do not wake

The mean discharge rates of cells recorded from the caudal region of the lesioned MVN was found to be 11.78 ± 1.08 spikes/s, n=33 cells at 4h, and 13.06 ± 1.45 spikes/s, n=30 cells at 6h. These values were not different than those recorded from the ureth + sham animals (12.92 ± 1.36 spikes/s, n=33cells, ANOVA on ranks $p>0.05$ Fig 4.2 Table 4.1).

As shown in figure 4.2, in the contralateral nucleus of ureth + UL animals no significant differences were observed in the mean discharge rates of MVN cells compared to ureth + sham animals (Table 4.1). These data indicate that when UL animals are kept anaesthetised for the 4 or 6 hours following surgery, thus not experiencing the stress associated with the lesion, no compensatory increase in the excitability of lesioned rostral MVN cells occurs.

Effect of the glucocorticoid agonist dexamethasone on the mean discharge rate of MVN neurones *in vitro*

As shown in figure 4.3, the mean discharge rate of MVN cells in nuclei from dexamethasone treated, sham-operated animals which remained anaesthetised for 4h following surgery (ureth + sham + dex) was not different from ureth + sham animals (see table 4.1), (rostral cells: ipsilateral MVN: 15.8 ± 1.59 spikes/s, n= 34 cells, contralateral MVN: 14.5 ± 1.5 spikes/s, n=40 cells or caudal cells ipsilateral MVN: 11.82 ± 1.7 spikes/s, n= 19 cells, contralateral MVN: 11.01 ± 2.10 spikes/s, n=11 cells, Fig 4.3 Table

4.1),. These data indicate that dexamethasone treatment alone does not result in changes in the mean *in vitro* discharge rate of MVN cells.

However, cells recorded from the rostral third of the lesioned nucleus of dexamethasone treated anaesthetised UL animals (ureth + UL + dex) animals were found to be firing significantly higher than the ureth + sham + dex treated group (22.31 ± 1.8 spikes/s, n=42 cells compared with 15.8 ± 1.59 spikes/s, n=74 cells, $p<0.005$ ANOVA on ranks Figure 4.3 Table 4.1). These data therefore indicate that while the lesion alone is not sufficient to induce the increase in excitability of MVN cells (figure 4.2) dexamethasone treatment to UL anaesthetised animals restores the large compensatory increase in excitability as seen in alert animals.

In the contralateral nucleus of ureth +UL + dex animals there were no differences seen in the mean discharge rate in either the rostral or caudal MVN cells (Table 4.1 and Figure 4.3) when compared with ureth + sham+ dex animals.

Effect of the glucocorticoid antagonist, RU38486, and the mineralcorticoid antagonist, spironolactone, on mean discharge rate of MVN neurones *in vitro*

In MVN slices prepared from animals which had been treated with the GR antagonist, RU38486, and labyrinthectomised under Avertin anaesthesia, the compensatory increase in excitability in rostral cells ipsilateral to the lesion was abolished (16.1 ± 1.5 spikes/s, n= 42 cells $p<0.01$ MWRST Figure 4.4), In contrast, rostral cells ipsilateral to the lesion from animals which had been treated with the MR antagonist, spironolactone, showed the same increase in excitability as cells in untreated alert UL animals (20.5 ± 1.4 spikes/s, n=31 cells, compared with 21.68 ± 1.6 spikes/s n=30 cells fig 4.5.). There were no differences in the mean discharge rate observed in either the caudal cells of the ipsilateral nucleus or in any region in the contralateral nucleus of either RU38486 or spironolactone treated groups figs 4.4, 4.5. Table 4.1

Summary

These data show that the compensatory increase in excitability seen at 4h post-UL in neurones in the rostral third of the ipsilateral MVN in alert UL animals was abolished when the animals were kept anaesthetised and did not experience the stress that normally follows UL. In accordance with this, in alert animals which were given the GR antagonist, RU38486, the compensatory increase in excitability was also abolished. Significantly, in urethane-anaesthetised animals administration of the synthetic GR agonist, dexamethasone, restored the compensatory increase in excitability. Therefore these experiments suggest that GR activation is necessary for VC to occur.

Treatment		Ipsilateral MVN spikes/s	Contralateral MVN spikes/s
4h anaesthetised -sham	rostral	14.86 ± 1.45	13.77 ± 1.24
	caudal	11.89 ± 1.47	13.15 ± 2.43
4h anaesthetised-UL	rostral	16.79 ± 1.49	14.69 ± 1.06
	caudal	11.78 ± 1.08	10.69 ± 1.57
6h anaesthetised-UL	rostral	16.98 ± 1.2	14.60 ± 1.34
	caudal	13.06 ± 1.45	10.69 ±0.97
4h anaesthetised-sham	rostral	15.8 ± 1.59	14.5 ± 1.5
+ dexamethasone	caudal	11.82 ± 1.7	11.01 ± 2.10
4h anaesthetised-UL	rostral	**22.31 ± 1.8**	16.25 ± 1.47
+ dexamethasone	caudal	12.17 ± 1.9	16.81 ± 2.45
4h post-UL recovery	rostral	**21.68 ± 1.6**	15.26 ± 1.41
	caudal	13.12 ± 1.76	15.91 ±1.61
4h post-UL recovery	rostral	16.1 ± 1.5	17.25 ± 1.8
+ RU38486	caudal	15.08 ± 1.69	13.95 ± 2.05
4h post-UL recovery	rostral	**20.5 ± 1.4**	16.79 ± 1.5
+ spironolactone	caudal	12.7 ± 1.62	16.32 ± 2.5

Table 4.1

The mean resting discharge rates of MVN neurones *in vitro* recorded from slices prepared from animals which had previously received a left labyrinthectomy or left sham surgery at the time stated. Values in bold indicate significantly different from corresponding control group.

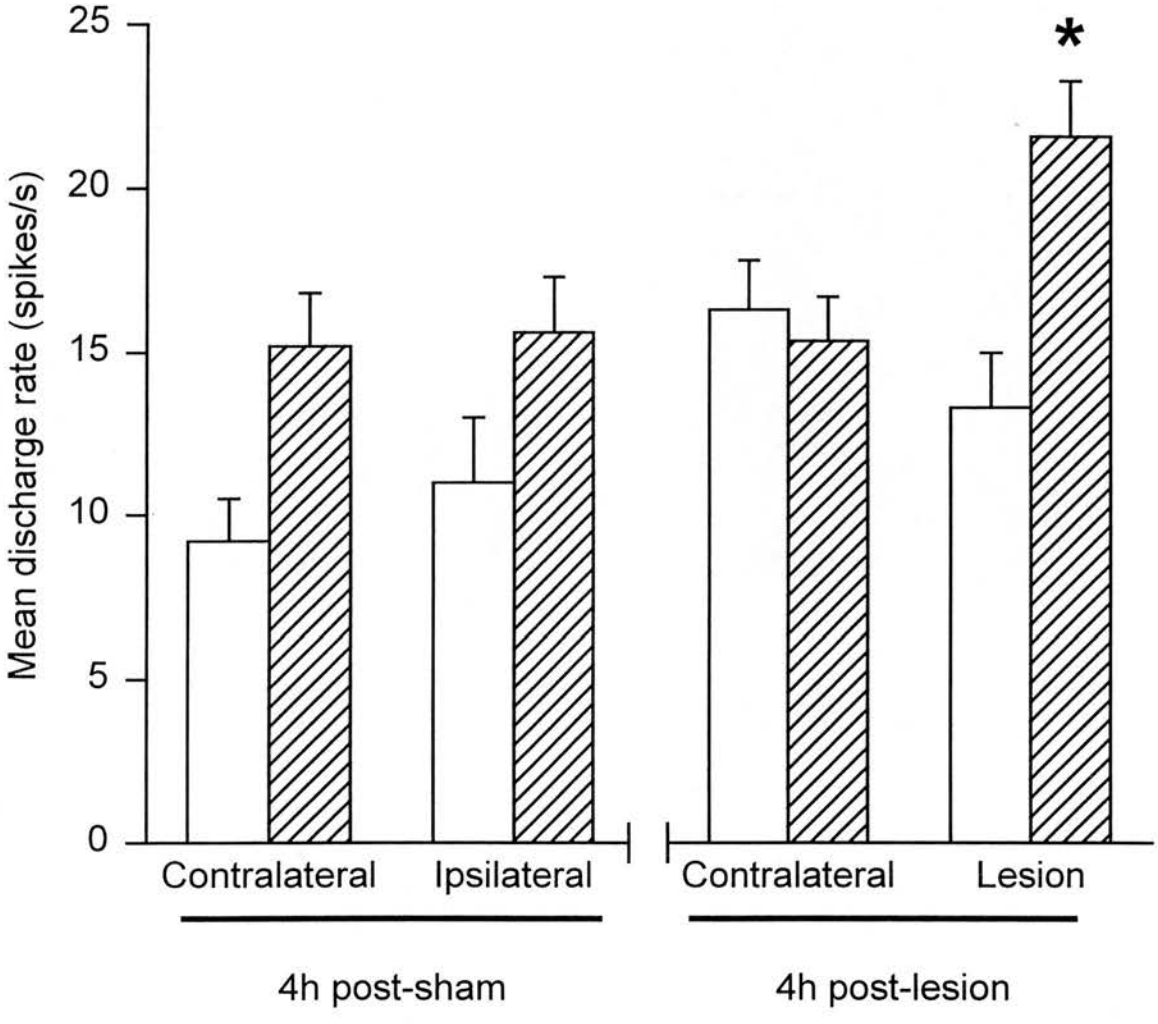

25
20
15
10
5
0
Mean discharge rate (spikes/s)
*
Contralateral
Ipsilateral
Contralateral
Lesion
4h post-sham
4h post-lesion

Figure 4.1

Histogram showing the mean ± s.e.m *in vitro* tonic discharge rates of MVN neurones in slices prepared from unilaterally-labyrinthectomised animals following 4h recovery after surgery.

Open bars represent data from cells recorded in the caudal third of the MVN

Hatched bars represent data from cells recorded in the rostral third of the MVN

Histograms represent the contralateral nuclei, sham-operated nucleus and lesioned nucleus 4h after surgery.

Statistics: *$p<0.0001$ compared to sham-operated rostral cells (ANOVA on ranks).

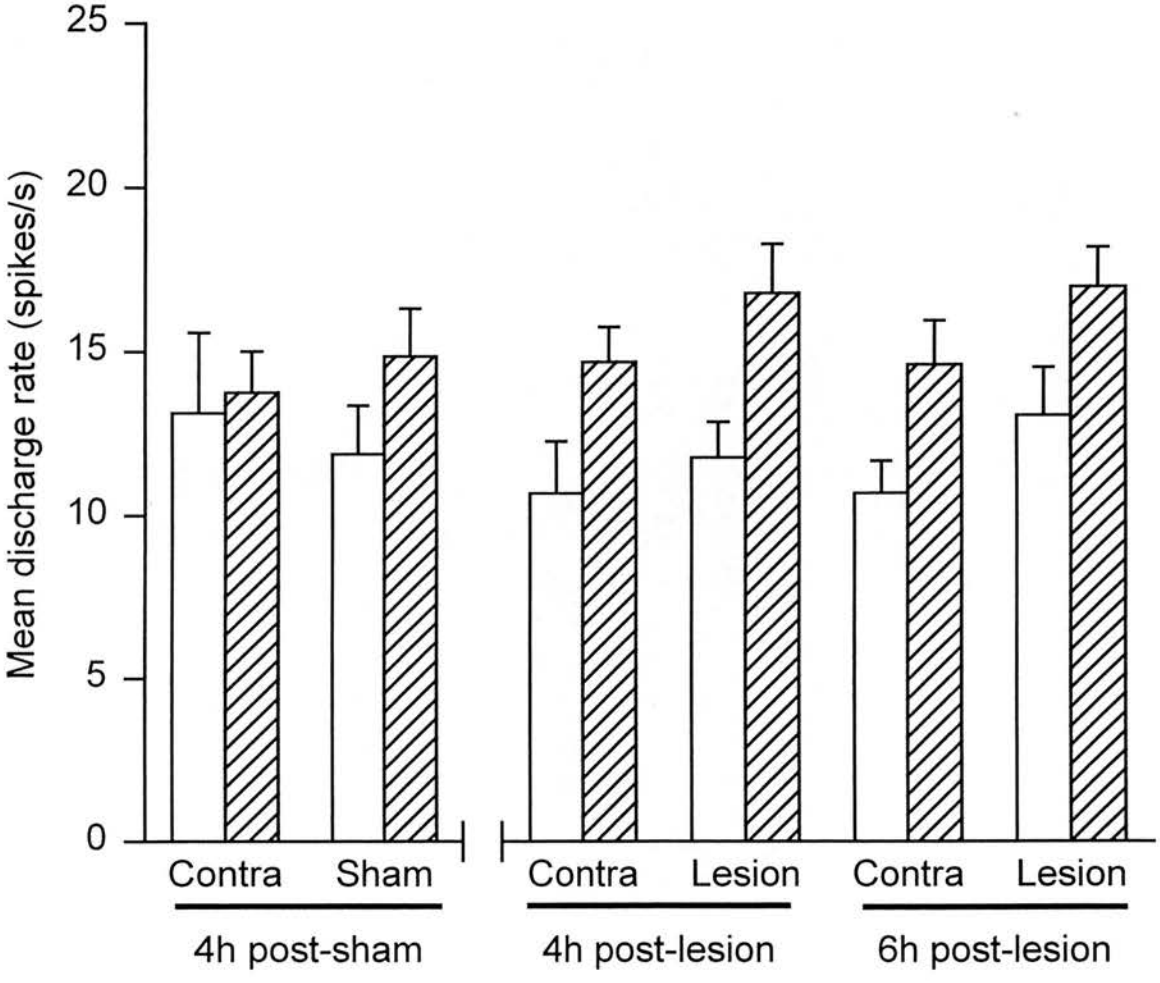

Mean discharge rate (spikes/s)
25
20
15
10
5
0
Contra
Sham
Contra
Lesion
Contra
Lesion
4h post-sham
4h post-lesion
6h post-lesion

Figure 4.2

Histogram showing the mean ± s.e.m *in vitro* tonic discharge rates of MVN neurones in slices prepared from unilaterally-labyrinthectomised animals which remained anaesthetised for 4h and 6h following surgery.

Histograms represent the contralateral nucleus (contra), the sham-operated nucleus (sham) or the lesioned nucleus (lesion) 4h or 6h after surgery.
Open bars represent data from cells recorded in the caudal third of the MVN
Hatched bars represent data from cells recorded in the rostral third of the MVN

No statistical differences were found between groups.

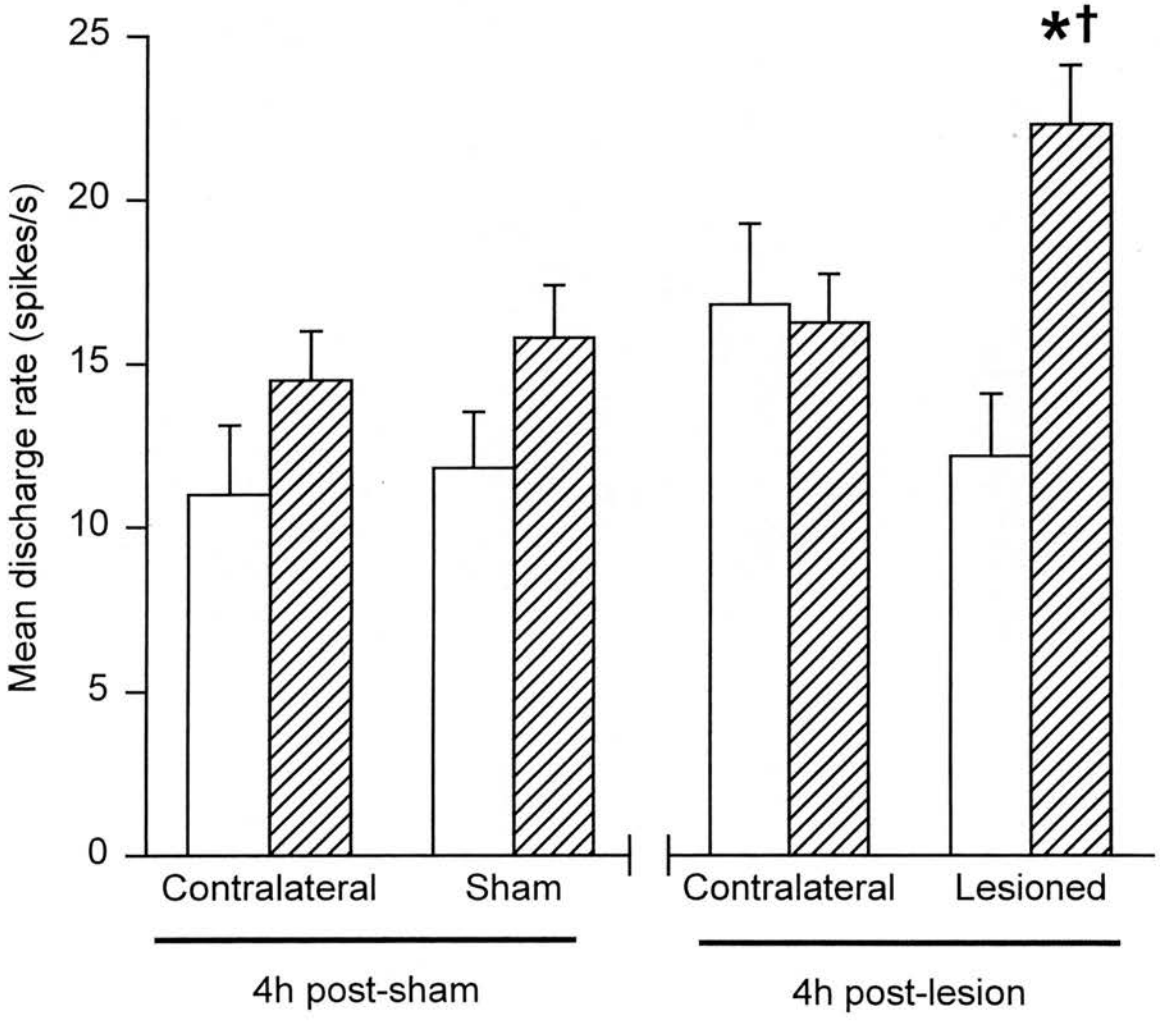

*†
25
20
15
10
5
0
Mean discharge rate (spikes/s)
Contralateral
Sham
Contralateral
Lesioned
4h post-sham
4h post-lesion

Figure 4.3

Histogram showing the mean ± s.e.m *in vitro* tonic discharge rates of MVN neurones in slices prepared from dexamethasone-treated unilaterally-labyrinthectomised animals which remained anaesthetised for 4h after surgery.

Histograms represent the contralateral nucleus, sham-operated nucleus or lesioned nucleus 4h after surgery. All animals were injected with dexamethasone (5mg/kg, i.p) 30 min prior to, and 2h after, surgery.
Open bars represent data from cells recorded in the caudal third of the MVN
Hatched bars represent data from cells recorded in the rostral third of the MVN

Statistics: *$p<0.0001$ compared to sham-operated dexamethasone-treated rostral MVN cells (ANOVA on ranks).
†$p<0.01$ compared to contralateral rostral MVN cells from urethane anaesthetised UL animals (ANOVA on ranks).

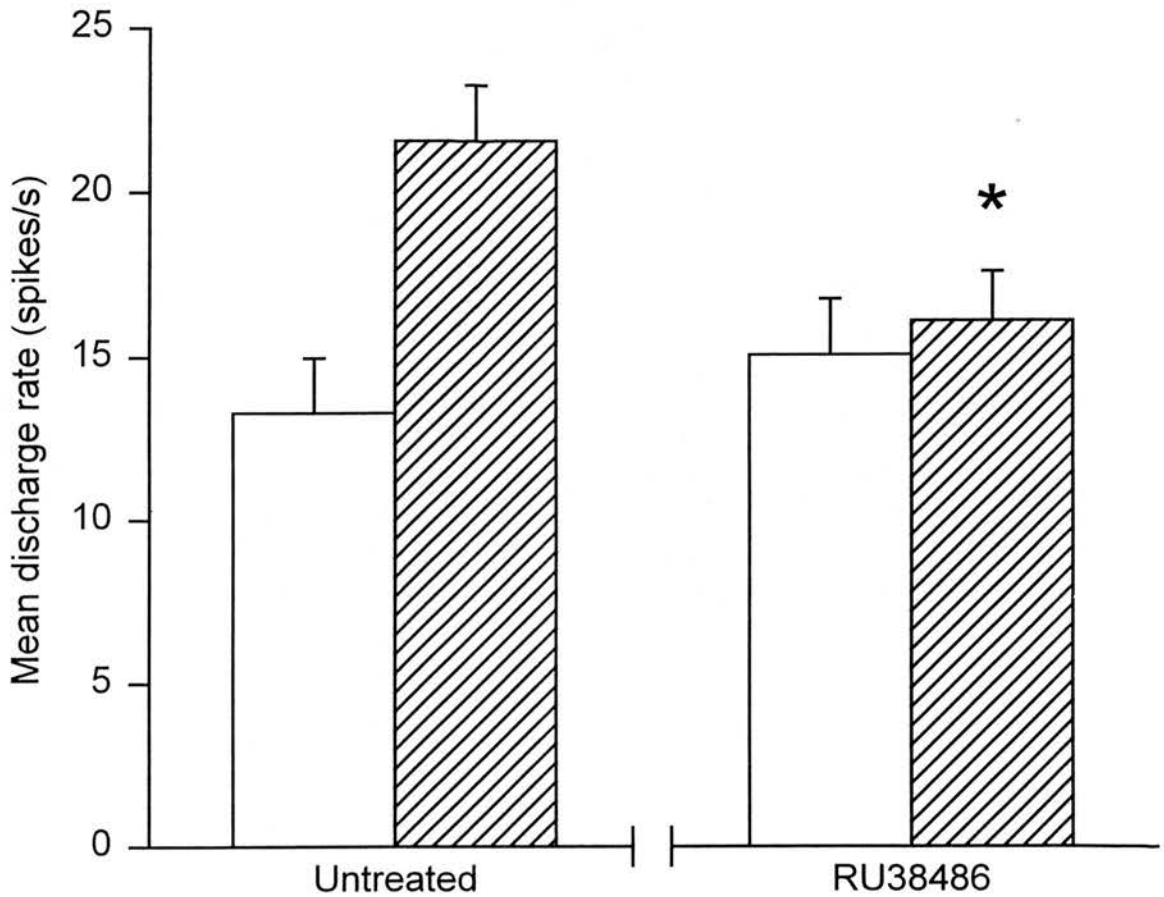

25
20
15
10
5
0
Mean discharge rate (spikes/s)
*
Untreated
RU38486

Figure 4.4

Histogram showing the mean ± s.e.m *in vitro* tonic discharge rates of MVN neurones in slices prepared from unilaterally-labyrinthectomised animals allowed to recover for 4h after surgery. The GR antagonist, RU38486, was administered (5mg/kg, i.m.) immediately prior to surgery.

Open bars represent data from cells recorded in the caudal third of the MVN
Hatched bars represent data from cells recorded in the rostral third of the MVN
Left hand columns represent data obtained from the lesioned nucleus from untreated UL animals.
Right hand columns represent data obtained from the lesioned nucleus of animals treated with RU38486.

Statistics: *$p<0.01$ compared to untreated rostral MVN cells (MWRST).

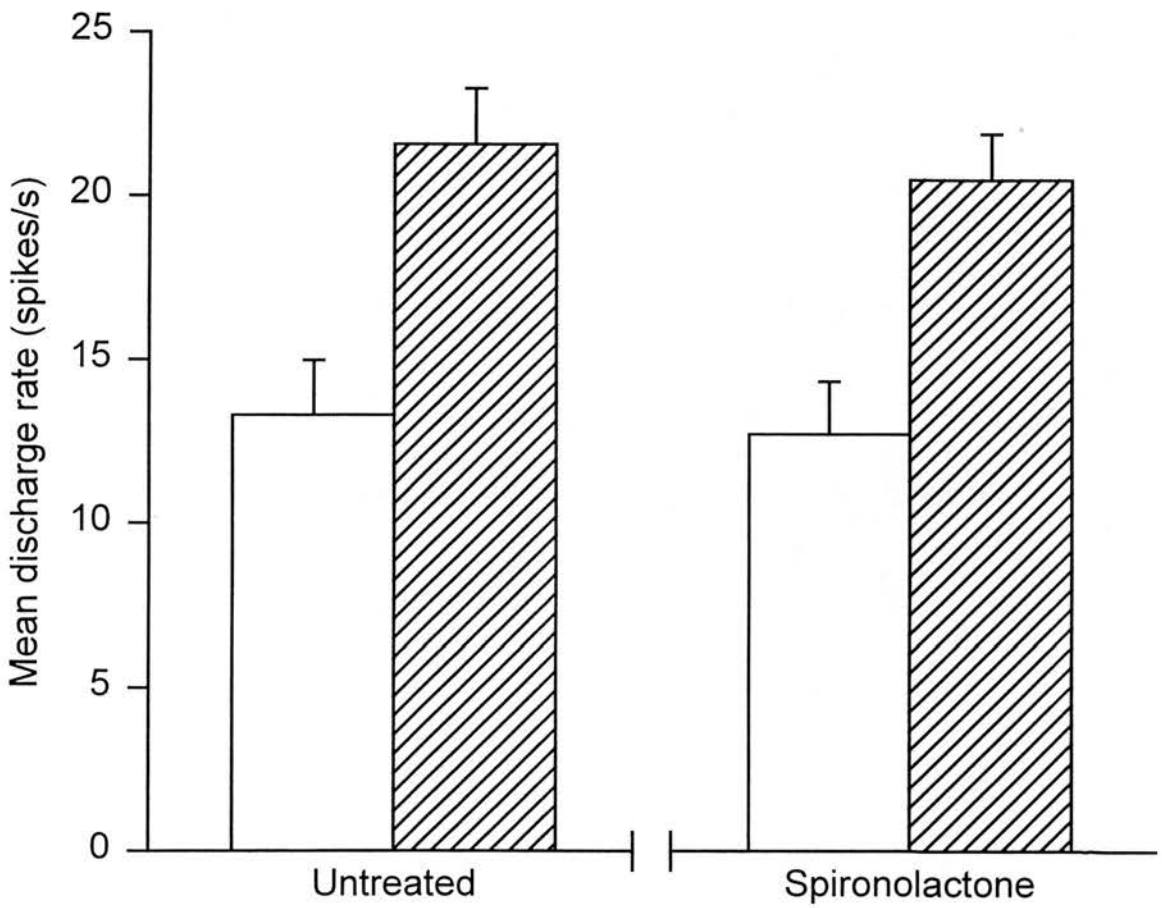

25
20
15
10
5
0
Mean discharge rate (spikes/s)
Untreated
Spironolactone

Figure 4.5

Histogram showing the mean ± s.e.m *in vitro* tonic discharge rates of MVN neurones in slices prepared from unilaterally-labyrinthectomised animals allowed to recover for 4h after surgery. The MR antagonist, spironolactone was administered (5mg/kg, i.p.) immediately prior to surgery

Open bars represent data from cells recorded in the caudal third of the MVN
Hatched bars represent data from cells recorded in the rostral third of the MVN
Left hand columns represent data obtained from the lesioned nucleus from untreated UL animals.
Right hand columns represent data obtained from the lesioned nucleus of animals treated with spironolactone.

Fos immunoreactivity

The number of Fos positive cells in the paraventricular nucleus was expressed as the number of positive cells per mm^2 and the group means are shown in figure 4.6. As can be seen in this figure and in figures 4.8, 4.10, 4.12 , in sham-operated rats there was a small, consistent amount of Fos-like immunoreactivity observed in the PVN at all time points studied (1.5h: 354.46 ± 75.5 cells/mm^2, 3h: 258.22 ± 24.23 cells/mm^2, and 6h: 226.24 ± 83.6 cells/mm^2) In animals which had received a labyrinthectomy there was a significant increase in Fos immunoreactivity in the PVN compared to sham-operated animals at 1.5 hours (686.22 ± 145.01 cells/mm^2, $P<0.05$ Students t-test fig 4.7) which was also observed at 3hours (552.06 ± 77.52 cells/mm^2 $P<0.005$ Students t-test fig 4.9), by 6 hours the amount of immunoreactivity had returned to basal levels (254.09 ± 125.48 cells/mm^2, $P<0.05$ students t-test fig 4.11). No significant difference was observed in the amount of immunoreactivity between the left and right nuclei. As many processes in the PVN are functionally coupled to activity in the supraoptic nuclei (SON) we also briefly examined Fos immunoreactivity in this region (Figs 4.7, 4.8, 4.9, 4.10,). Although, due to technical limitations, it was not possible to fully quantify the number of Fos positive cells in the SON, sections from all treated animals were examined. A moderate level of Fos immuno-positive cells was observed in the SON of UL animals at 1.5 and 3 h following UL, very little or no Fos positive cells were observed in sham-operated animals at the same time points.

Behavioural observations

To investigate whether the changes seen in the intrinsic excitability of rostral MVN neurones after UL are correlated with the behavioural recovery, we used circular walking as an index. As shown in figure 3.3 the occurrence of circular walking is high in the early stages of recovery but decreases as VC occurs. In vehicle treated animals the incidence of circular walking towards the lesioned side during the 2-5 hours post-UL was high, followed by a significant decrease at 6h and 7h post-UL ($P<0.001$ one-way RM ANOVA). In animals pre-treated with the

glucocorticoid agonist, dexamethasone, no significant differences were seen at any time over the recording period compared with vehicle treated controls. ($p>0.05$ two-way RM ANOVA), Figure 4.13.

However, in animals which were pre-treated with the glucocorticoid antagonist, RU38486, before labyrinthectomy under Avertin anaesthesia, differences were observed in the rate of behavioural recovery. Initially these animals were observed to be less active and inquisitive than vehicle treated animals. This observation could be due to a number of possibilities, firstly these animals may not have recovered from the anaesthetic fully when behavioural recordings were made, secondly they may have a decreased level of alertness, both of which may account for the lack of movement observed in the first 3 hours post-UL.

At later time points (6 and 7 h post-UL), the RU38486 treated animals showed a similar rate of circular walking to that observed at 4 and 5 hours post-UL, at 6 and 7 hour time points this was found to be significantly higher when compared to vehicle treated animals ($p<0.05$ two-way RM ANOVA). Figure 4.14

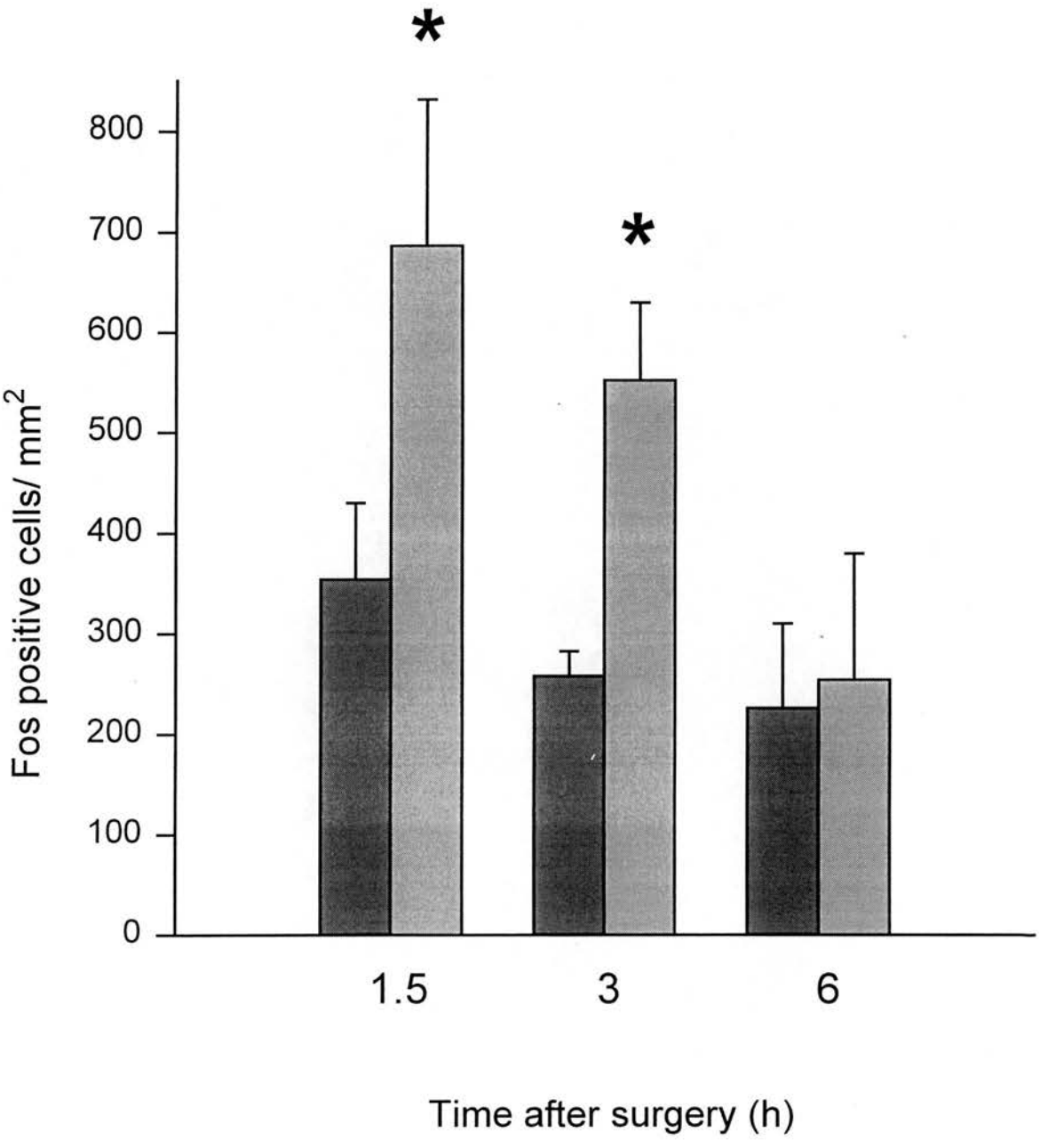

*
*
800
700
600
500
400
300
200
100
0
Fos positive cells/ mm2
1.5
3
6
Time after surgery (h)

Figure 4.6

Histogram showing the mean ± s.e.m number of Fos-positive cells/mm^2 in the PVN at varying times (1.5h, 3h, 6h) after unilaterally-labyrinthectomised or sham surgery.

Dark grey bars represent data from sham-operated animals.
Light grey bars represent data from unilaterally-labyrinthectomised animals.

Statistics: *$p<0.005$ compared to 1.5h sham-operated animals (t-test).
*$p<0.05$ compared to 3h sham-operated animals (t-test).

Figure 4.7

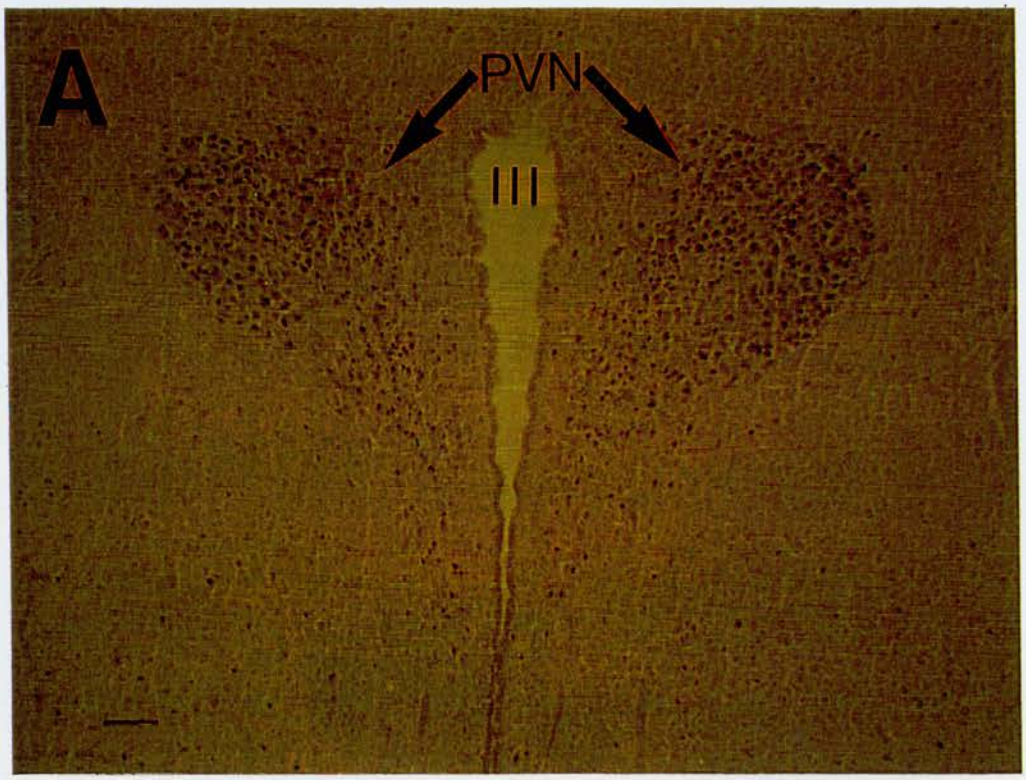
Photomicrographs of coronal hypothalamic sections (15μm) showing Fos immunoreactivity (darkly-stained nuclei) 1.5 hours after recovery from unilateral labyrinthectomy.

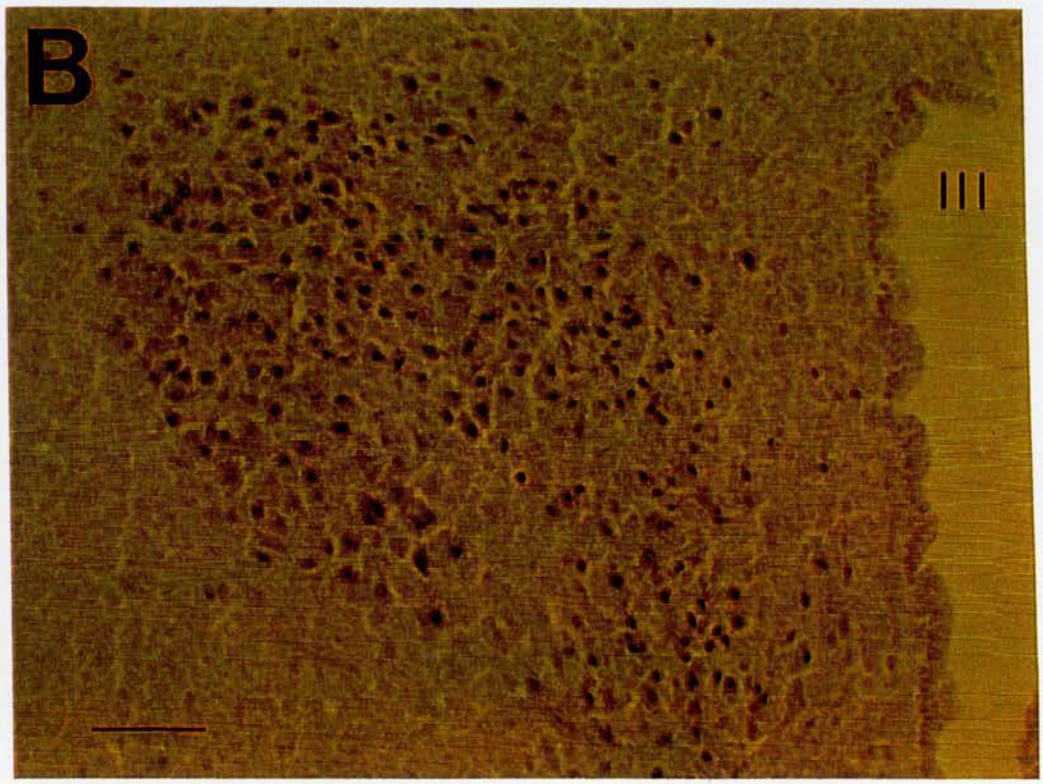
A. Low power photomicrograph of the paraventricular nuclei (PVN), showing Fos immunoreactivity (III: third ventricle: scale bar= 100μm).

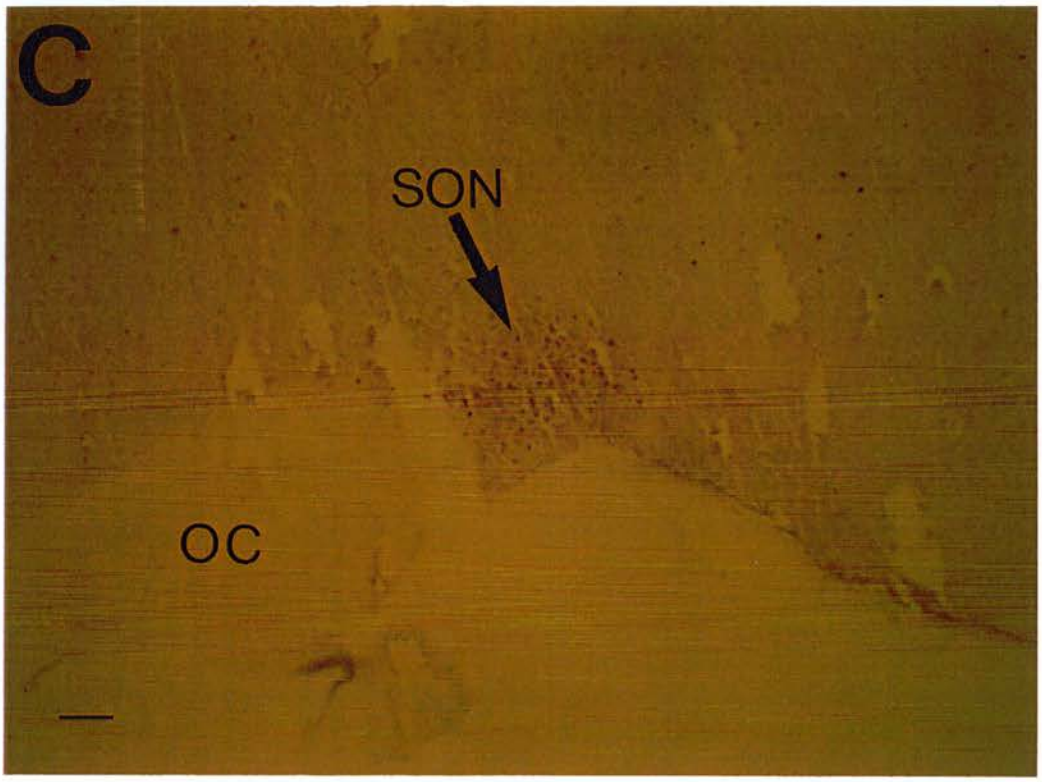
B. High power photomicrograph from the same section as (A), showing Fos immunoreactivity in the left PVN. Note the high levels of Fos immunoreactivity throughout the whole extent of the nucleus (III: third ventricle: scale bar=100μm).

C. Low power photomicrograph of a supraoptic nucleus (SON), from the same animal as (A), showing Fos immunoreactivity (OC: optic chiasm: scale bar=100μm).

D. High power photomicrograph from the same section as (C), showing Fos immunoreactivity in a SON. Note the moderate levels of Fos immunoreactivity throughout the whole extent of the nuclei (OC: optic chiasm scale bar=100μm).


A
PVN
III


B
III


C
SON
OC


D
OC

Figure 4.8

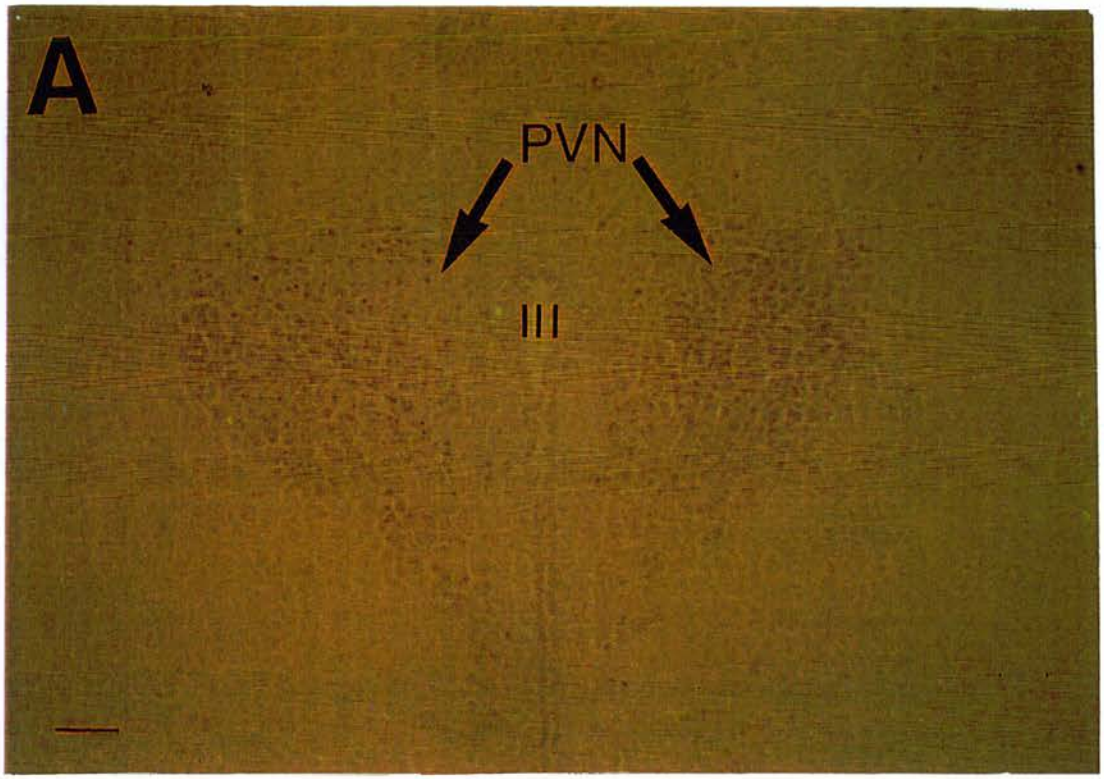
Photomicrographs of coronal hypothalamic sections (15μm) showing Fos immunoreactivity (darkly-stained nuclei) 1.5 hours after recovery from sham surgery

A. Low power photomicrograph of the paraventricular nuclei (PVN), showing Fos immunoreactivity (III: third ventricle: scale bar=100μm).

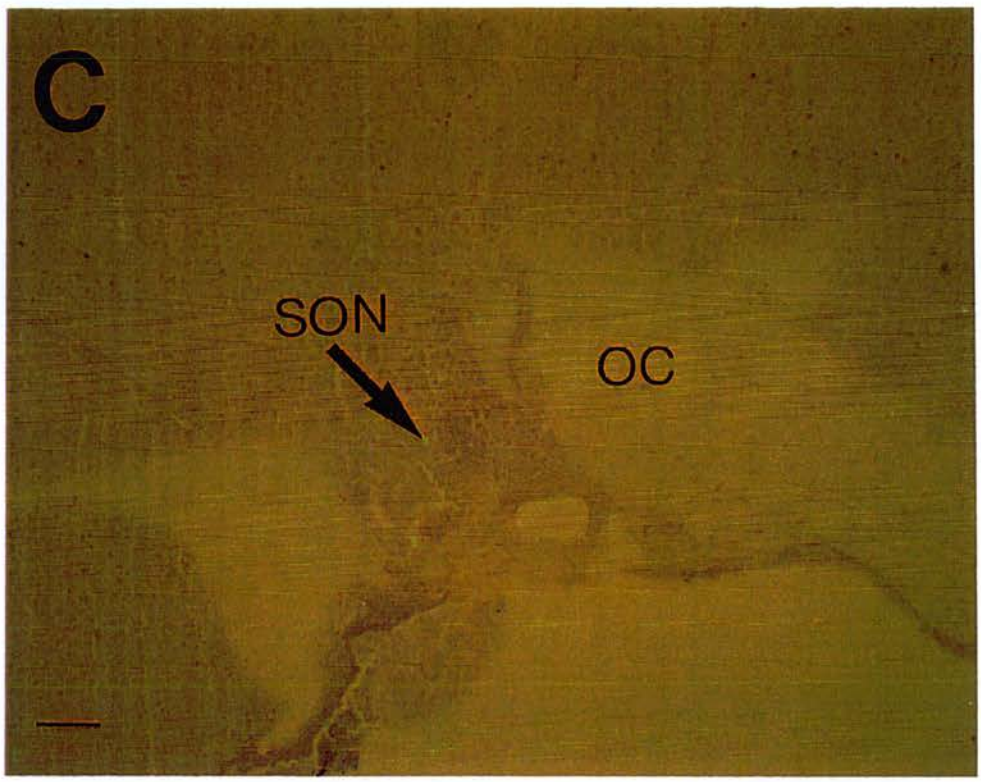
B. High power photomicrograph from the same section as (A), showing Fos immunoreactivity in the left PVN. Note the low levels of Fos immunoreactivity throughout the whole extent of the nucleus (III: third ventricle: scale bar=100μm).

C. Low power photomicrograph of a supraoptic nucleus (SON), from the same animal as (A), stained for Fos immunoreactivity (OC: optic chiasm: scale bar=100μm).

D. High power photomicrograph from the same section as (C), stained for Fos immunoreactivity in a SON. (OC: optic chiasm scale bar=100μm).


A
PVN
III


B
III


C
SON
OC

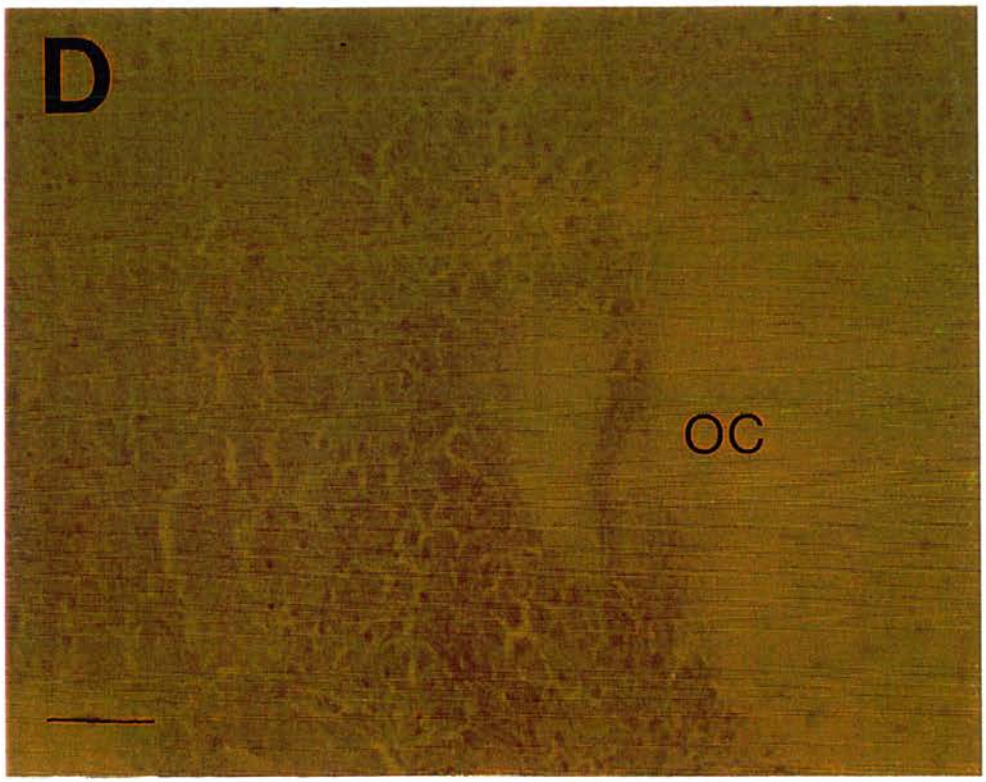

D
OC

Figure 4.9

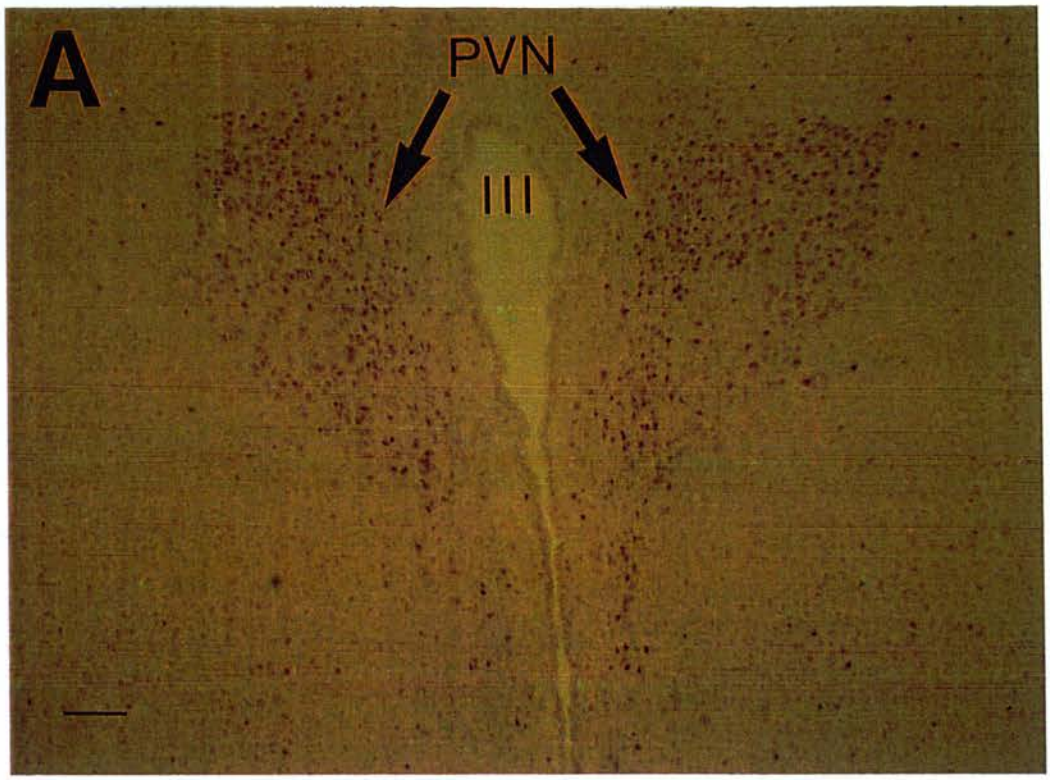
Photomicrographs of coronal hypothalamic sections (15μm) showing Fos immunoreactivity (darkly-stained nuclei) 3 hours after recovery from unilateral labyrinthectomy.

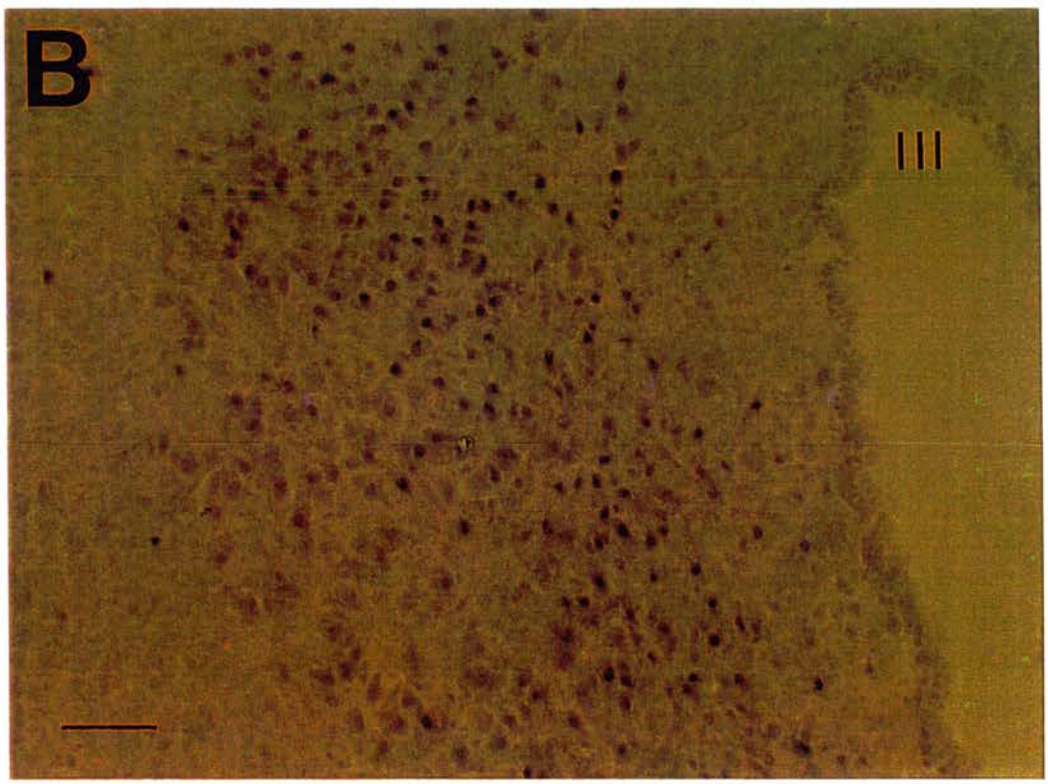
A. Low power photomicrograph of the paraventricular nuclei (PVN), showing Fos immunoreactivity (III: third ventricle: scale bar=100μm).

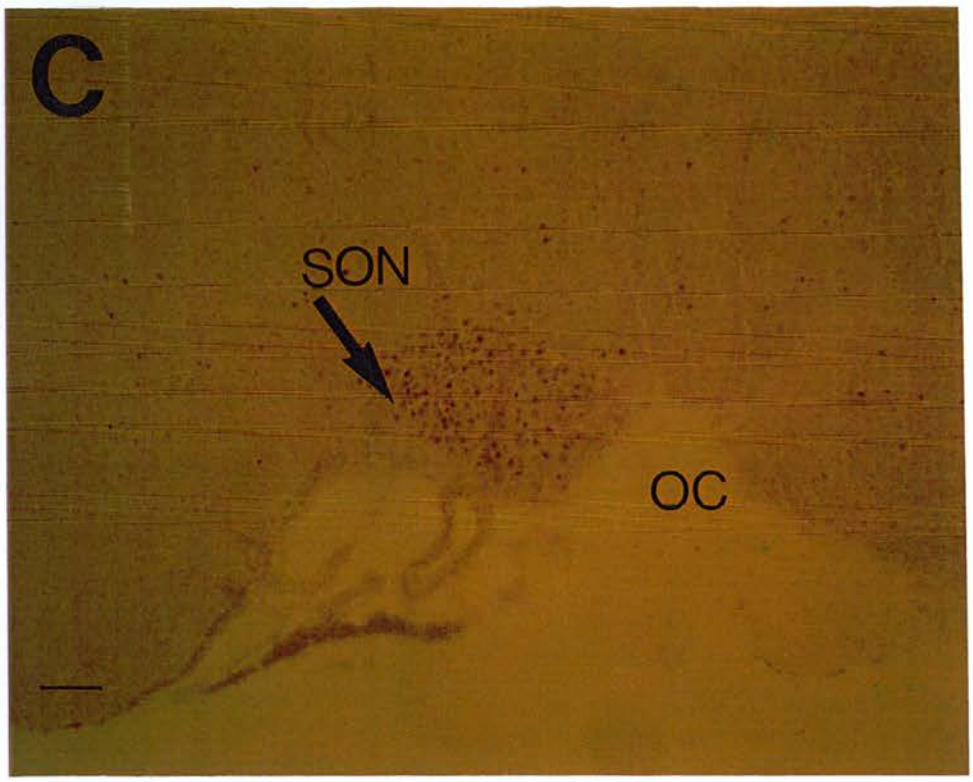
B. High power photomicrograph from the same section as (A), showing Fos immunoreactivity in the left PVN. Note the high levels of Fos immunoreactivity throughout the whole extent of the nucleus (III: third ventricle: scale bar=100μm).

C. Low power photomicrograph of a supraoptic nucleus (SON), from the same animal as (A), showing Fos immunoreactivity (OC: optic chiasm: scale bar=100μm).

D. High power photomicrograph from the same section as (C), showing Fos immunoreactivity in a SON. Note the moderate levels of Fos immunoreactivity throughout the whole extent of the nuclei (OC: optic chiasm scale bar=100μm).


A
PVN
III


B
III


C
SON
OC

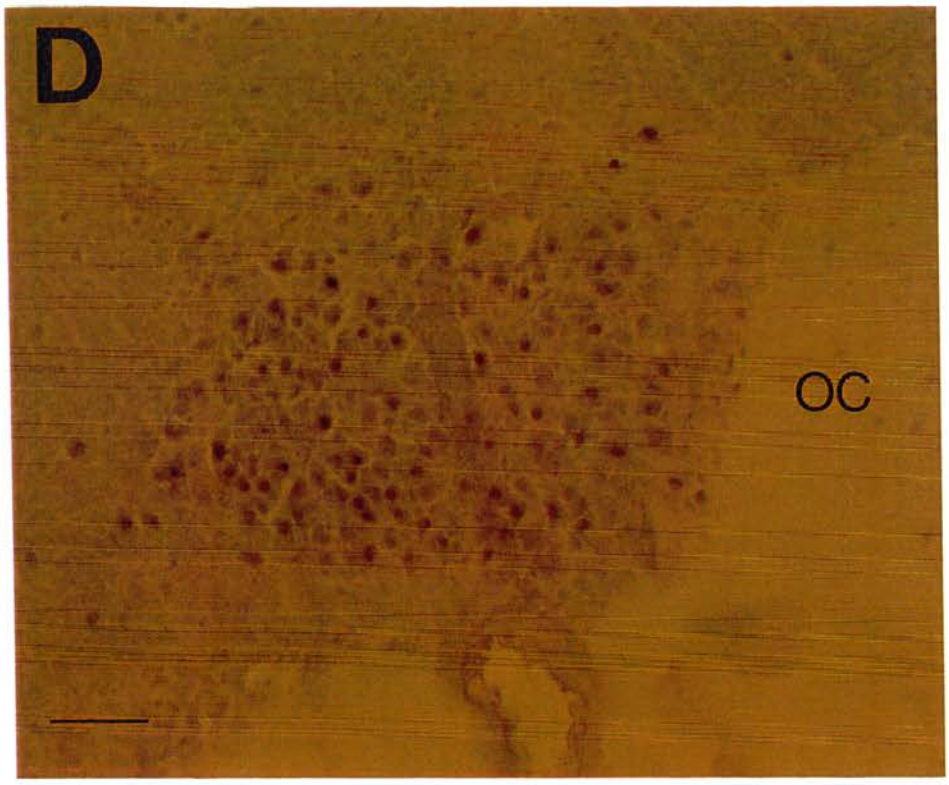

D
OC

Figure 4.10

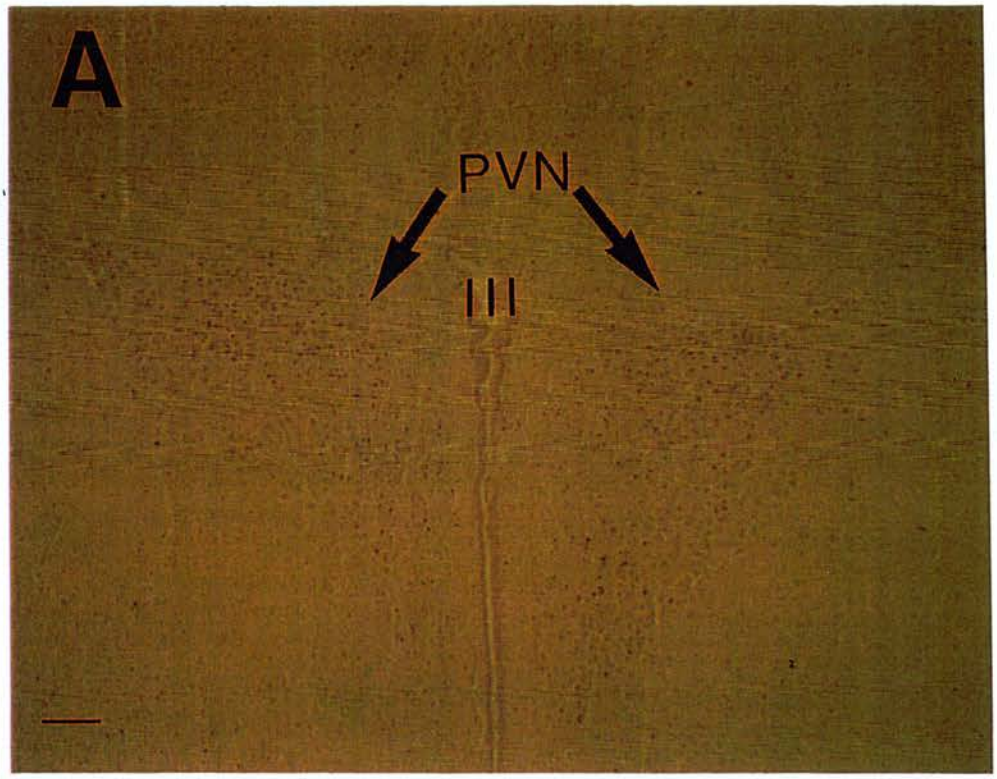
Photomicrographs of coronal hypothalamic sections (15μm) showing Fos immunoreactivity (darkly-stained nuclei) 3 hours after recovery from sham surgery

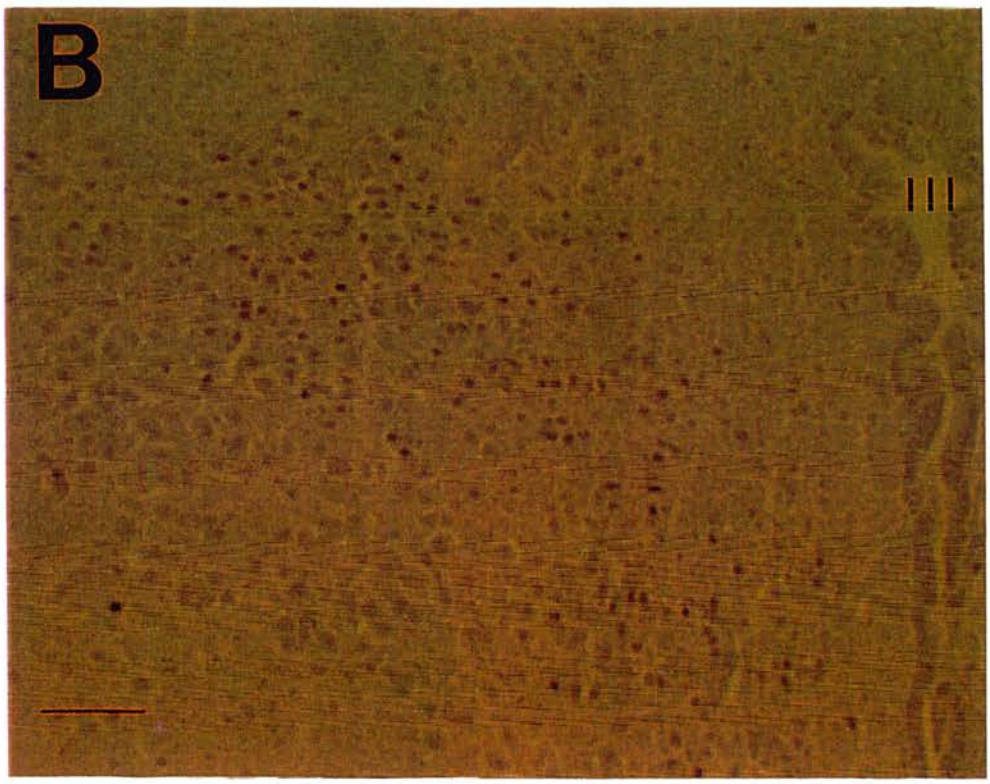
A. Low power photomicrograph of the paraventricular nuclei (PVN), showing Fos immunoreactivity (III: third ventricle: scale bar=100μm).

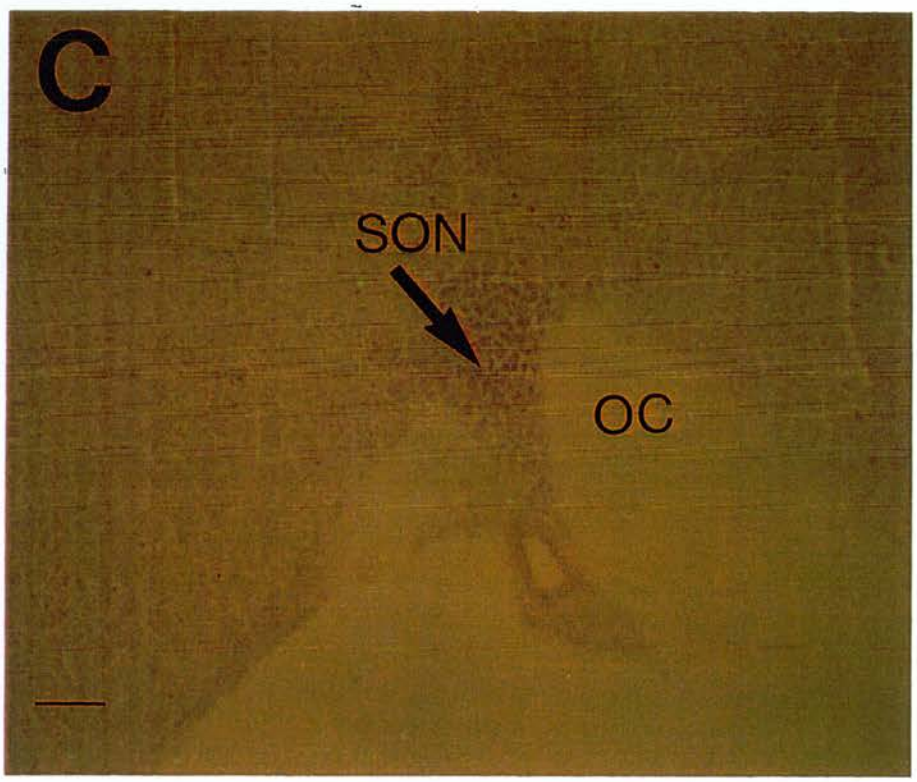
B. High power photomicrograph from the same section as (A), showing Fos immunoreactivity in the left PVN. Note the low levels of Fos immunoreactivity throughout the whole extent of the nucleus (III: third ventricle: scale bar=100μm).

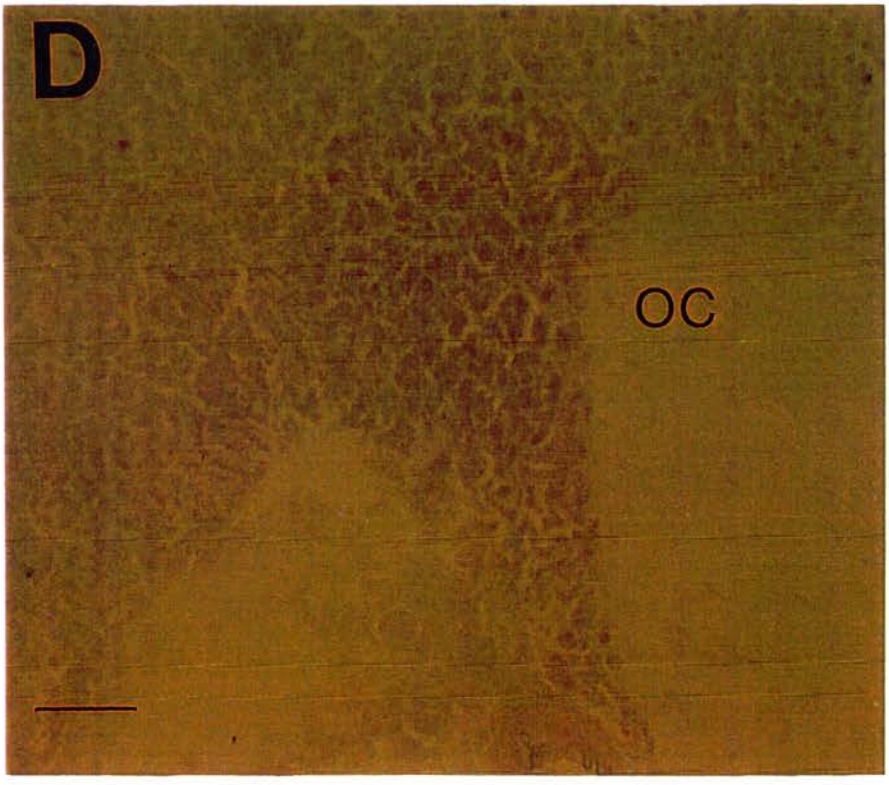
C. Low power photomicrograph of a supraoptic nucleus (SON), from the same animal as (A), stained for Fos immunoreactivity (OC: optic chiasm: scale bar=100μm).

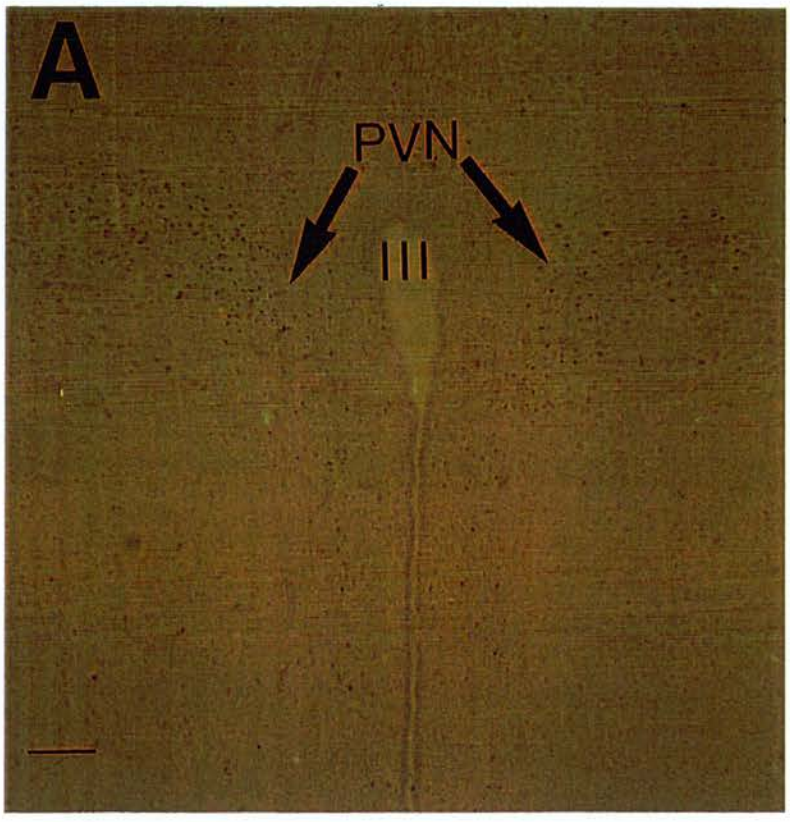
D. High power photomicrograph from the same section as (C), stained for Fos immunoreactivity in a SON. (OC: optic chiasm scale bar=100μm).


A
PVN
III


B
III


C
SON
OC


D
OC


A
PVN
III

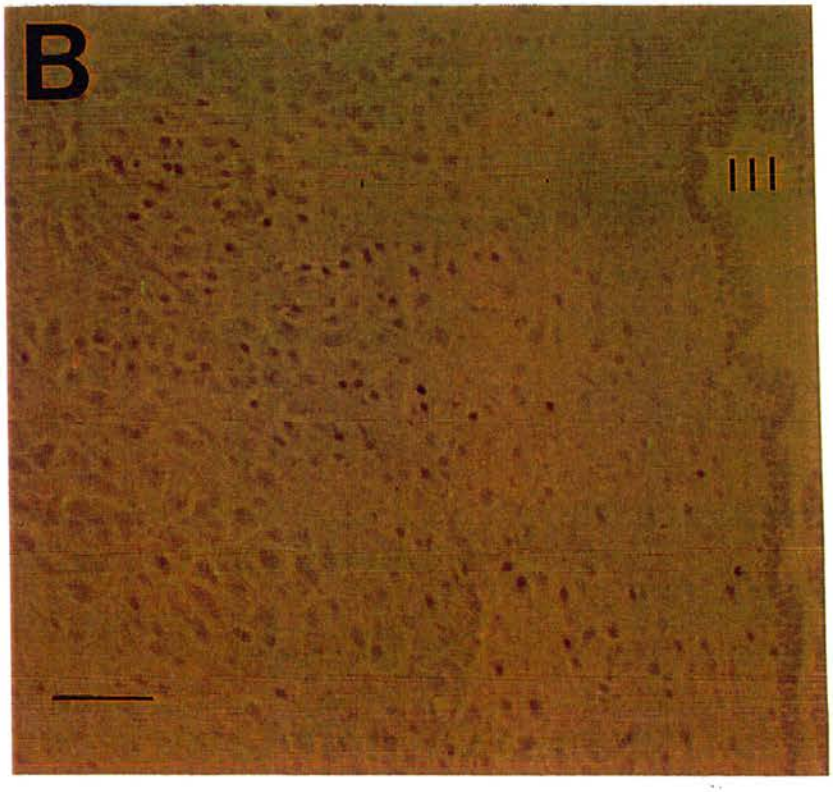
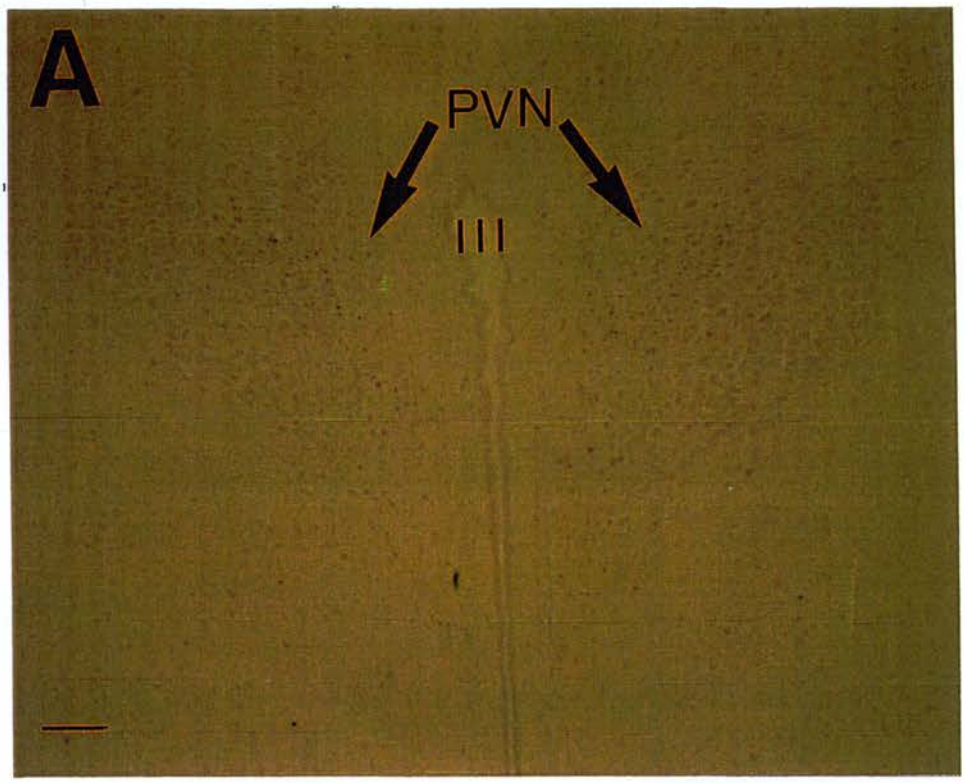

B
III

Figure 4.11

Photomicrographs of coronal hypothalamic sections (15μm) showing Fos immunoreactivity (darkly-stained nuclei) 6 hours after recovery from unilateral labyrinthectomy.

A. Low power photomicrograph of the paraventricular nuclei (PVN), showing Fos immunoreactivity (III: third ventricle: scale bar=100μm).

B. High power photomicrograph from the same section as (A), showing Fos immunoreactivity in the left PVN. Note the low levels of Fos immunoreactivity throughout the whole extent of the nucleus (III: third ventricle: scale bar=100μm).


A
PVN
III

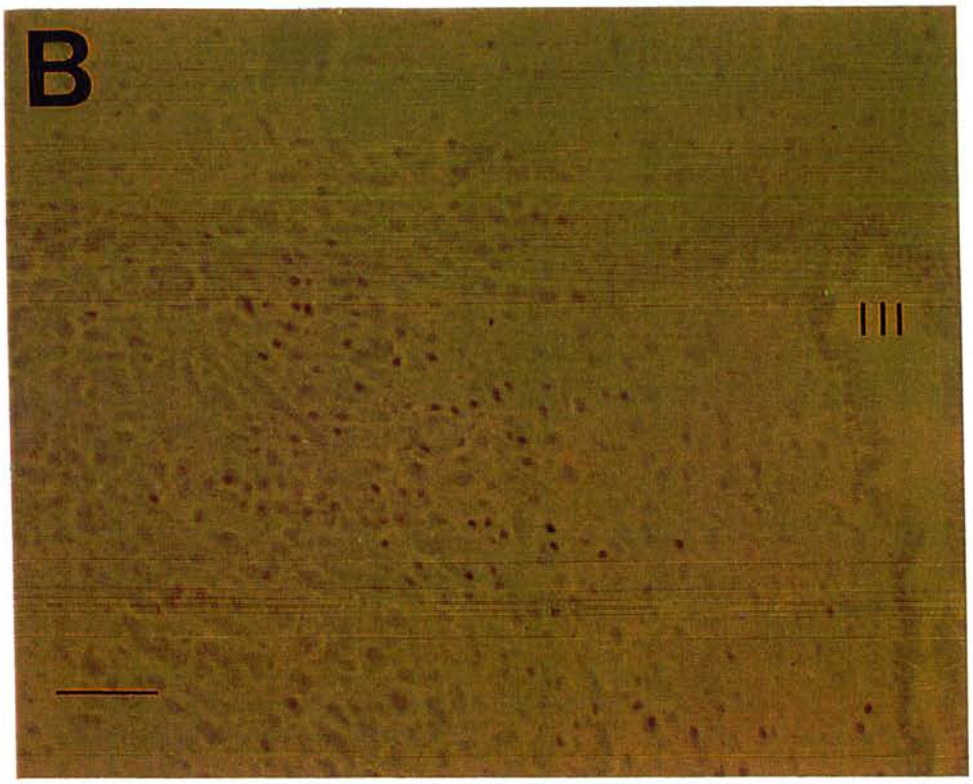

B
III

Figure 4.12

Photomicrographs of coronal hypothalamic sections (15μm) showing Fos immunoreactivity (darkly-stained nuclei) 6 hours after recovery from sham surgery.

A. Low power photomicrograph of the paraventricular nuclei (PVN), showing Fos immunoreactivity (III: third ventricle: scale bar=100μm).

B. High power photomicrograph from the same section as (A), showing Fos immunoreactivity in the left PVN. Note the low levels of Fos immunoreactivity throughout the whole extent of the nucleus (III: third ventricle: scale bar=100μm).

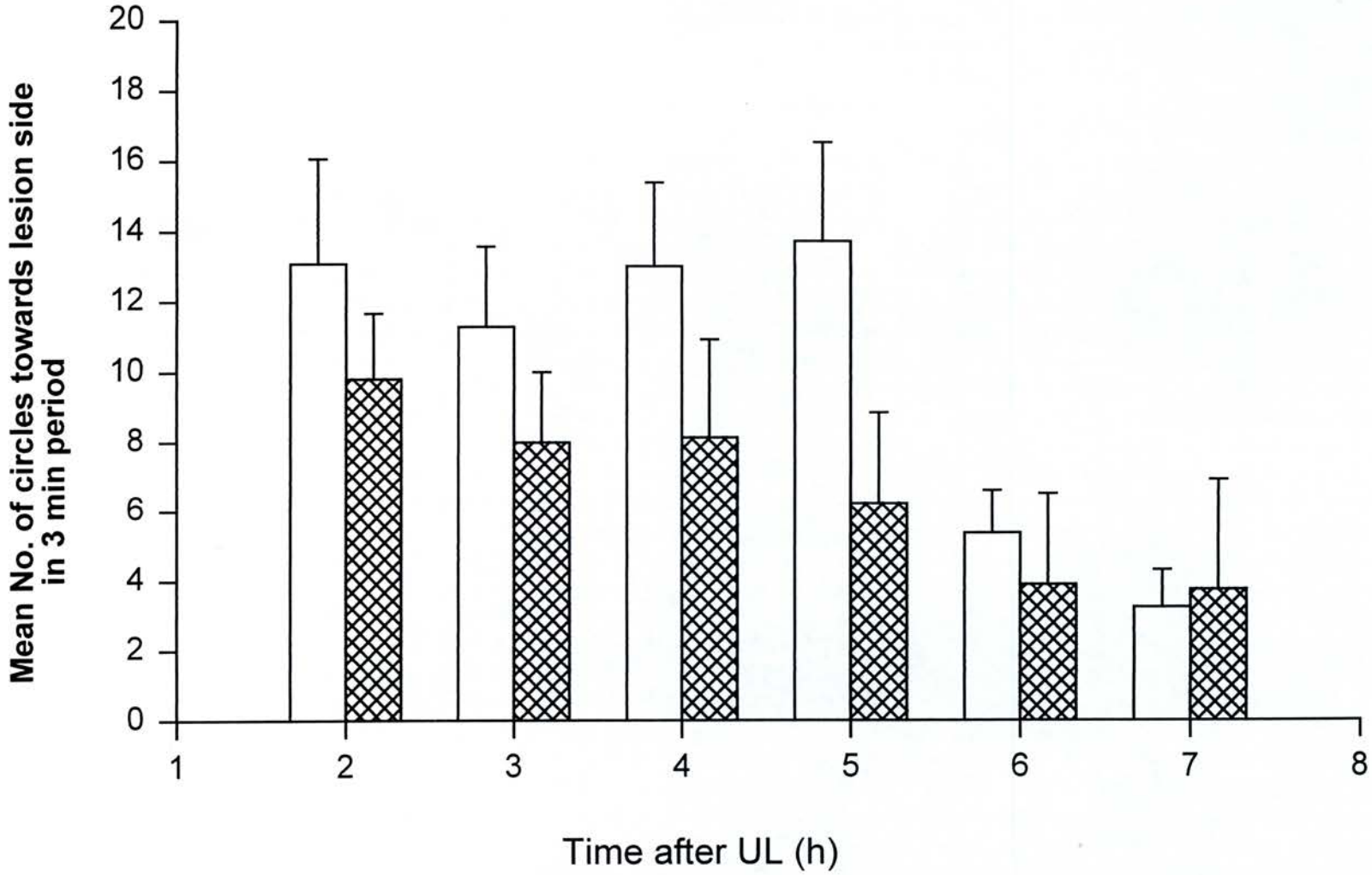

Mean No. of circles towards lesion side in 3 min period
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
Time after UL (h)

Figure 4.14

Animals received a left UL under Avertin anaesthesia. Circular walking was elicited by placing the animals in an open enclosure, and the number of circles in 3x 3 min periods following a 1 minute contról period was calculated. Data shown is the mean ± s.e.m. for each group of animals at the appropriate time point.

Hatched bars correspond to data obtained from dexamethasone treated unilaterally labyrinthectomised animals.
Open bars correspond to data obtained from vehicle treated unilaterally labyrinthectomised animals.

No significant differences were found between groups.

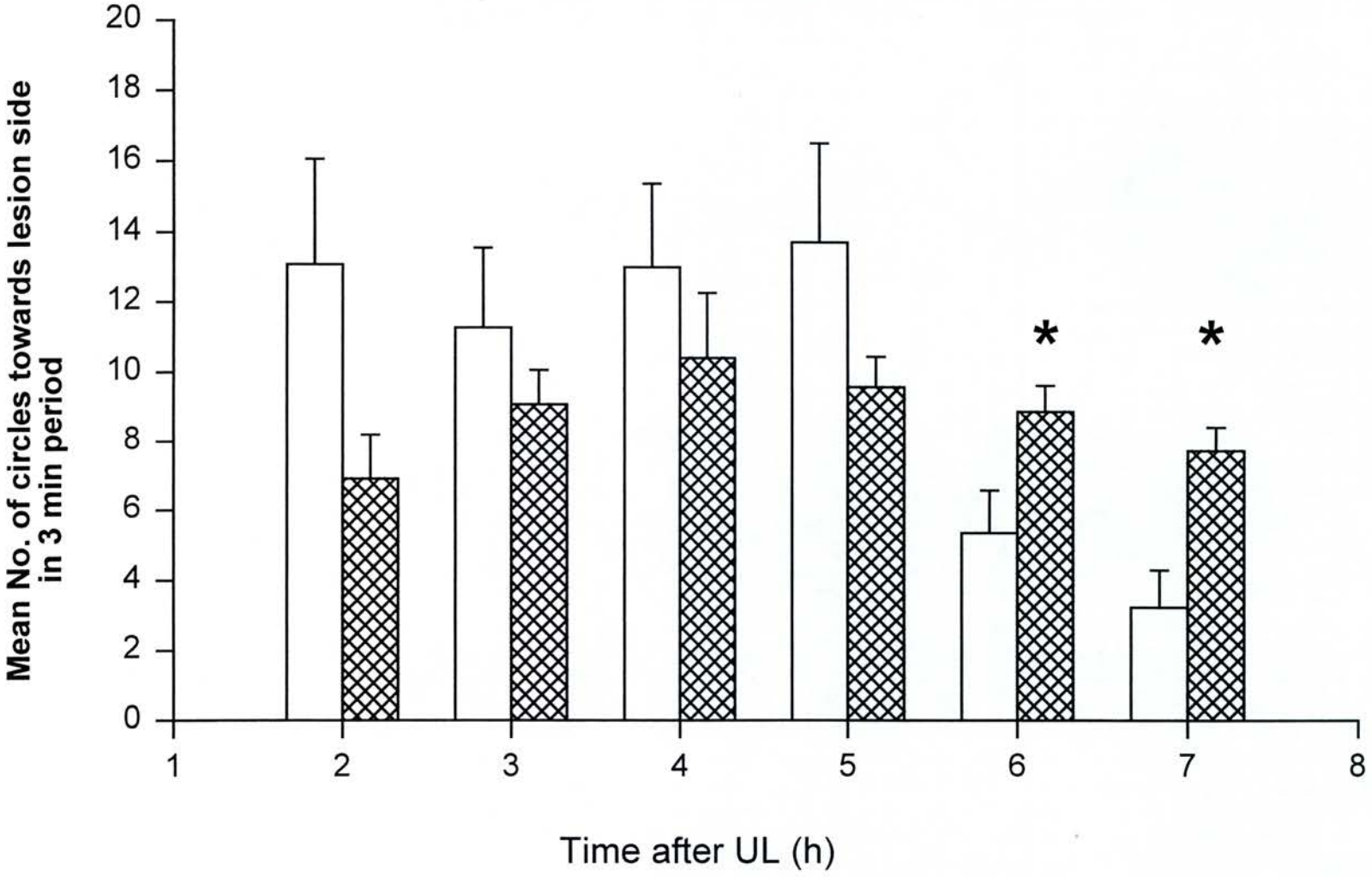

Mean No. of circles towards lesion side in 3 min period
20
18
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
7
8
*
*
Time after UL (h)

Figure 4.15

Animals received a left UL under Avertin anaesthesia. Circular walking was elicited by placing the animals in an open enclosure, and the number of circles in 3x 3 min periods following a 1 minute control period was calculated. Data shown is the mean ± s.e.m. for each group of animals at the appropriate time point.

Hatched bars correspond to data obtained from RU38486 treated unilaterally labyrinthectomised animals.
Open bars correspond to data obtained from vehicle treated unilaterally labyrinthectomised animals.

Statistics: $p<0.05$ two-way RM ANOVA

4.6 DISCUSSION

The compensatory increase in excitability observed in rostral neurones of the lesioned MVN following UL was abolished in animals which were not allowed to recover from anaesthesia. Administration of the glucocorticoid agonist, dexamethasone, to mimic the activation of the stress axis in non-recovery animals restored the increase in excitability to rostral MVN neurones in the lesioned nucleus. In contrast, administration of the glucocorticoid antagonist, RU38486, abolished the increase in excitability and delayed the behavioural recovery as shown be the persistence of circular walking at later time points. The mineralcorticoid antagonist, spironolactone, had no effect on the discharge rate of rostral cells in these animals. These experiments suggest that acute activation of the stress axis, the subsequent release of endogenous glucocorticoids and the activation of GR receptors are necessary to induce the compensatory increase in excitability observed in rostral cells of the lesioned nucleus and inducing the behavioural recovery during the early stages of VC.

Further evidence that the stress axis is strongly activated after UL was provided by the fact that there was a significant increase in Fos immunoreactivity bilaterally in the PVN during the first 3h following UL which returned to basal levels 6h post UL in animals allowed to recover from anaesthesia. A similar result was obtained by Cirelli *et al.* (1996) who showed a bilateral increase in c-Fos mRNA levels in the PVN of animals which were culled 3h after UL.

These results are in accordance with those of Yamanaka *et al.* (1995) who reported that RU38486 delayed normal behavioural recovery following UL when measuring spontaneous nystagmus as an index of recovery. However, Alice *et al.* (1998), demonstrated that in guinea pigs none of the three doses of dexamethasone administered (5, 10, or 40 mg/kg i.p.) at 0, 12, 24 and 36 hours following UL resulted in a significant increase in the compensation of SN. The reason for the discrepancies between these studies may lie in the fact that in the guinea pig there are a series of radical differences in various components of the pituitary-adrenal axis compared with

other species. It is known that guinea pigs have very high circulating levels of cortisol, and low-affinity, low capacity transcortin so that free cortisol levels are relatively higher (Keightley *et al.*, 1991). Guinea pigs also have very low-affinity glucocorticoid receptors, with approximately one-twentieth the affinity for dexamethasone as those in the rodent (Kraft *et al.*, 1979). Administration of dexamethasone to guinea pigs may therefore have little effect on the rate of compensation as it is likely that the majority of glucocorticoid receptors are normally occupied in this species.

Genomic action of glucocorticoids

Although fast membrane actions of glucocorticoids and neurosteroids have been described (see Joëls, 1997; Zakon, 1998 for reviews) it is unlikely that in present experiments such an action could account for the increase in excitability seen in lesioned rostral MVN neurones 4h post UL. The fact that at two hours post-UL there was no change in the mean discharge rate of MVN neurones, suggests that the compensatory increase in the mean discharge rate develops somewhere between 2 and 4 hours and is due to a slower genomic response by these neurones. The available data indicates that voltage gated Na, K and Ca conductances and Ca-dependent K conductances, may be targets for glucocorticoid actions (Joëls, 1997; Zakon, 1998). All of these conductances are important in generating the pacemaker like conductances of MVN neurones (Serafin et al., 1991a,b; Johnston *et al.,* 1994). It is possible then, that activation of intracellular GR receptors in the lesioned rostral MVN neurones results in changes in the intrinsic membrane properties of MVN neurones which subsequently results in the compensatory increase in intrinsic excitability. Intracellular studies of the rostral MVN neurones of the lesioned nucleus are now required to establish if changes in the intrinsic properties of these cells occur. It will also be important to establish more accurately when the increase in excitability of rostral MVN neurones occurs. This would open up a whole range of experiments to investigate the second messenger pathways which may participate in this process. If a genomic action is required for this process, this could be

investigated by experiments with protein synthesis inhibitors. By administering the inhibitor at different time intervals it could be established if new proteins are manufactured during the compensatory increase in excitability and the time frame in which this event occurs.

Membrane actions of glucocorticoids

Although it is unlikely that fast membrane actions of glucocorticoids are involved in inducing the increase in excitability of rostral lesioned MVN neurones as discussed above, however it is conceivable that there is a rapid action by both glucocorticoids and the neurosteroids to modulate GABA receptors. As demonstrated in chapter 3, the functional efficacy of both $GABA_A$ and $GABA_B$ receptors is altered in all areas of the bilateral MVN following UL. Rapid actions by steroids and neurosteroids on GABA receptors in the MVN have previously been described. Yamamoto *et al.* (1998), recently showed that the naturally occurring neurosteroid, dehydroepiandosterone, which is secreted from the adrenal cortex during stressful stimuli (Corpechot *et al.* 1981), blocked the decrease in discharge rate of MVN neurones to microiontophoretically applied GABA. In the labyrinthectomised animal there is an immediate increase in the amount of GABA reaching the lesioned nucleus from the commissural inhibitory pathway (Thompson *et al.* 1986). One early response to counteract the imbalance in excitability between the two MVN may therefore be by neurosteroids and glucocorticoids acting on $GABA_A$ receptors within the lesioned MVN to decrease the enhanced inhibition from the commissural and/or cerebellar pathways.

It is possible that two mechanisms of glucocorticoid action participate synergistically in the process of vestibular compensation. Firstly, glucocorticoids and neurosteroids may have a rapid membrane action, binding to specific sites on $GABA_A$ receptors to modulate their activity. Secondly, activation of intracellular GR may result in changes in the intrinsic membrane properties of MVN neurones which then result in the compensatory increase in intrinsic excitability. In future

experiments these effects could be examined in adrenalectomised (ADX)-UL rats. If these mechanisms are important in inducing the behavioural recovery following UL, then ADX-UL rats would show slower rates of VC than controls, there may be no compensatory increase in excitability of rostral lesioned MVN neurones and there may also be differences in the efficacy of GABA receptors from those seen in UL rats.

Location of action

Although the results of the present study suggest that activation of glucocorticoid receptors and a subsequent genomic response are obligatory for the expression of lesion-induced plasticity in MVN neurones, it is not clear where the glucocorticoids are acting. GR receptors are expressed widely in the CNS by neurones and glia. High levels of the receptor are expressed in the hippocampus, neocortex, cerebellum, thalamus and stress-related nuclei of the hypothalamus and brainstem (Funder and Sheppard, 1987; Reul and DeKloet, 1985; Reul *et al.*, 1989; Arronson *et al.*, 1988; Ahima and Harlan, 1990). Although glucocorticoids are essential for the process of vestibular compensation, their action may not be directly on the MVN itself. Glucocorticoids may be acting on areas of the vestibulocerebellum to modulate the GABAergic input from this system to the MVN. They may also be acting back on the HPA axis to regulate the amount of stress-related compounds released. To establish if the glucocorticoid action is in fact in the MVN, experiments currently in progress in our laboratory are investigating the effects of incubating a slice containing a single MVN for 4h in the specific $GABA_A$ agonist, muscimol, and dexamethasone, in an attempt to mimic the results described in chapter 3, i.e. the compensatory increase in excitability of rostral MVN neurones and the down-regulation of GABA receptors in the MVN. To date, it appears that these experimental conditions are sufficient to induce the functional down-regulation of $GABA_A$ receptors but not to induce the increase in mean discharge rate (Him and Dutia, unpublished observations). These preliminary observations suggest that the action of endogenous glucocorticoids in the labyrinthectomised animals may not be

in the MVN or, that other compounds and events are involved in inducing the increase in excitability seen in lesioned rostral MVN neurones.

It may not be endogenous glucocorticoids which result in the increase in excitability seen in rostral lesioned MVN neurones, but other molecules secreted in response to stressful stimuli. For example, both ACTH and CRF are released in the cascade of events following stress, and although they ultimately lead to an increase in the release of glucocorticoids from the adrenal cortex, both these compounds have been shown to have independent actions on other regions of the brain (see section 4.2 for a discussion).

However, in the urethane-anaesthetised UL animals administration of dexamethasone restored the increase in excitability of rostral MVN neurones. Dexamethasone will also act on the HPA axis to decrease the amounts of CRF and ACTH released, and so it seems unlikely that the compensatory increase in excitability is due to actions by CRF or ACTH. However this remains to be investigated.

Summary

These results provide electrophysiological, behavioural and morphological evidence supporting a role for the stress axis in vestibular compensation and indicate that the activation of glucocorticoid receptors is necessary for the expression of lesion-induced plasticity in MVN neurones. Therefore the acute stress that normally follows vestibular lesions appears to be important in inducing behavioural recovery.

The exact actions of glucocorticoids in inducing the compensatory increase in excitability of rostral MVN neurones in the lesioned nucleus is unknown and future experiments are now required in order to establish where and how glucocorticoids are acting. Changes in Fos immunoreactivity in the PVN should now be investigated further with the use of double labelling techniques for oxytocin, vasopressin and CRF to determine which cell types are activated following UL.

RESUME AND FUTURE EXPERIMENTS

In **Chapter 3** it was demonstrated that there was a significant and sustained increase in the mean resting discharge rate of MVN neurones within 4 hours of unilateral labyrinthectomy in the rat, specifically of the cells in the rostral third of the MVN, where the lesioned afferents predominantly terminate. This increase in *in vitro* discharge rate remained elevated until 1 week after surgery. It is probable that this increase in excitability of rostral lesioned MVN cells may be important in the recovery of resting discharge of the vestibular neurones ipsilateral to the lesion, as it would overcome the excessive commissural inhibition from the intact side.

It was suggested that the increase in excitability was possibly due to the down-regulation of GABA receptors on the MVN cells of the lesioned side, in response to the sustained commissural inhibition which these cells are subject to following UL. This hypothesis was tested by preparing slices from animals 4h after UL and testing the responsiveness of MVN cells in both the ipsilateral and contralateral sides to the $GABA_A$ agonist, muscimol and the $GABA_B$ agonist, baclofen. The results from these experiments confirmed that there is a marked decrease in the responsiveness of MVN cells in the lesioned nucleus to both $GABA_A$ and $GABA_B$ agonists. Although this could account for the increase in excitability seen *in vitro*, less marked changes in the responsiveness of MVN cells to GABA agonists also occurred in the caudal region of the lesioned nucleus and the contralateral nucleus, but without corresponding changes in the mean discharge rate of cells in these regions. These results suggest the possibility that other, currently unidentified mechanisms are important in the generating the increase in discharge rate following UL. Future experiments should aim to determine how long the changes in GABA receptor efficacy persists after UL, and if this functional down-regulation in rostral cells is accompanied by changes in $GABA_A$ receptor subunit expression. There are few examples of $GABA_B$ receptor down-regulation in other systems and it is possible that the rapid changes in $GABA_B$ receptor function described in these experiments represent the first demonstration of such a regulation of $GABA_B$ function. The process of vestibular compensation may

therefore not only become a useful tool for studying the mechanisms of plasticity in the CNS but also for investigating the intracellular signalling pathways which are involved in the rapid functional down-regulation of $GABA_A$ and $GABA_B$ receptor subtypes in mammalian neurones.

It is possible that there are changes in the ionic conductances which generate the resting discharge of MVN neurones *in vitro* after UL. Intracellular studies are now required in order to determine if there are changes in the membrane conductances of lesioned rostral MVN cells.

In **Chapter 4** it was demonstrated that the significant increase in excitability of rostral MVN neurones ipsilateral to the lesion was abolished if animals remained anaesthetised for the 4 or 6 hours following UL, and also in animals which had been allowed to recover following UL but had been pre-treated with the glucocorticoid antagonist RU38486. In anaesthetised animals which had been treated with the glucocorticoid agonist, dexamethasone, the significant increase in excitability of rostral MVN cells was restored. Together these experiments suggest that acute activation of the hypothalamo-pituitary-adrenal stress axis, the subsequent release of endogenous glucocorticoids and the activation of GR receptors are necessary to induce the compensatory increase in excitability in MVN cells. This was further supported by the fact that there was strong activation of the stress axis during the first 3h following UL, as demonstrated by a significant increase in Fos immunoreactivity in the PVN of the hypothalamus.

It is likely that a genomic response by the lesioned rostral MVN neurones is necessary to induce the compensatory increase in excitability as it was expressed somewhere between 2 and 4 h following UL. Future experiments are now necessary to establish the exact time course over which this increase in excitability develops. Once this is established it will be possible to investigate the second messenger pathways and molecular events which are involved in this process.

The site of glucocorticoid action in VC still remains to be established. Preliminary experiments suggest that glucocorticoids may not be acting directly in the MVN to induce the increase in excitability and so future experiments should aim to establish where the primary action of these compounds is and other pathways which may be involved.

In conclusion, the results demonstrate for the first time, that changes in the cellular properties of MVN neurones occur rapidly after UL and suggest that at least two mechanisms are important in inducing vestibular compensation: the activation of GR receptors by endogenous glucocorticoids and rapid changes in the efficacy of GABAergic inhibition. Although this work has provided answers to some of the outstanding questions regarding vestibular compensation, as always, this experimental work has raised more questions than it has answered, and provided many avenues for future exploration.

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